

08/11/00
JCS93 U.S. PTO

08-14-00

A

NEW UNITED STATES UTILITY PATENT APPLICATION
under 37 C.F.R. 1.53(b)

CERTIFICATE OF EXPRESS MAILING UNDER 37 C.F.R. § 1.10: The undersigned hereby certifies that this United States Patent Application and all papers noted herein as being attached, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service under 37 C.F.R. § 1.10 today, August / / , 2000 and is addressed to: Box: Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

Assistant Commissioner for Patents
Patent Applications
Washington, D.C. 20231

EXPRESS MAIL NO. EL591995113US
Kathleen M. Williams
Kathleen M. Williams

JCS93 U.S. PTO
09/03/00
08/11/00

Enclosed herewith is a new patent application and the following papers:

First Named Inventor (or application identifier): **Zheng, Chao-Feng**

Title of Invention: **Compositions and Methods Utilizing Stable Reporter Cell Lines for Detection of Pathway-Specific Signal Transduction**

08/11/00
JCS93 U.S. PTO

6.
7.

- Specification 81 pages (including specification, claims, abstract) / 42 claims (7 independent)
- Declaration/Power of Attorney is:
 - attached in the regular manner (unexecuted).
 - ☐ NOT included, but deferred under 37 C.F.R. § 1.53(f).
- 17 Distinct sheets of ☐ Formal ☒ Informal Drawings
- ☐ Preliminary Amendment.
- ☐ Information Disclosure Statement
 - ☐ Form 1449
 - ☐ A copy of each cited prior art reference
- 6. ■ Assignment with Cover Sheet (Unexecuted).
- 7. ■ Priority is hereby claimed under 35 U.S.C. § 119 based upon the following application(s):

Country	Application Number	Date of Filing (day, month, year)
US	60/150,902	26/08/99

☐ Priority is hereby claimed under 35 U.S.C. § 120 based upon the following application(s):

Country	Application Number	Date of Filing (day, month, year)

- 8. ☐ Priority document(s).
- 9. ■ Statement Claiming Small Entity Status (Unexecuted).

NEW UNITED STATES UTILITY PATENT APPLICATION
under 37 C.F.R. 1.53(b)

Page 2

Atty. Docket No.25436/1510

10. ☐ Microfiche Computer Program (Appendix).
11. ☐ Nucleotide and/or Amino Acid Sequence Submission.
☐ Computer Readable Copy.
☐ Paper Copy (identical to computer copy).
☐ Statement verifying identity of above copies.

12. Calculation of Fees:

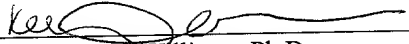
FEES FOR	EXCESS CLAIMS	FEE	AMOUNT DUE
Basic Filing Fee (37 C.F.R. § 1.16(a))			\$690.00
Total Claims in Excess of 20 (37 C.F.R. § 1.16(c))	22	18.00	\$396.00
Independent Claims in Excess of 3 (37 C.F.R. § 1.16(b))	4	82.00	\$328.00
Multiple Dependent Claims (37 C.F.R. § 1.16(d))		.00	\$0.00
Subtotal - Filing Fee Due			\$1,414.00
	REDUCE BY (%) (\$)		
Reduction by 50%, if Small Entity (37 C.F.R. §§ 1.9, 1.27, 1.28)	0.50		\$1414.00
TOTAL FILING FEE DUE			\$0.00
Assignment Recordation Fee (if applicable) (37 C.F.R. § 1.21(h))	0	40.00	\$0.00
GRAND TOTAL DUE			\$707.00

13. PAYMENT is:
- ☒ included in the amount of the GRAND TOTAL by our enclosed check. A general authorization under 37 C.F.R. § 1.25(b), second sentence, is hereby given to credit or debit our Deposit Account No. 19-0733 for the instant filing and for any other fees during the pendency of this application under 37 C.F.R. §§ 1.16, 1.17 and 1.18.
- ☐ not included; will be paid with response to Notice of Missing Parts.

14. All correspondence for the attached application should be directed to:

PALMER & DODGE, LLP
One Beacon Street
Boston, Massachusetts 02108
Telephone: (617) 573-0451
Facsimile: (617) 227-4420

Date: 8/11/00
Reg. No. 34,380

By: 
Kathleen M. Williams, Ph.D

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant or Patentee: Chao-Feng Zheng
Serial No.: Not Assigned Yet
Filed: Concurrent Herewith
Entitled: "Composition and Methods Utilizing Stable Reporter Cell Lines for Detection of Pathway-Specific Signal Transduction"
Attorney Docket No.: 25436/1510

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(d) AND 1.27(d)) -SMALL BUSINESS CONCERN**

I hereby declare that I am an official empowered to act on behalf of the small business concern identified below:

Name of Organization: **Stratagene**
Address of Organization: **11011 North Torrey Pines Road
La Jolla, CA 92037**

I hereby declare that the small business concern identified above qualifies as a small business concern as defined in 37 C.F.R. § 1.9(d) for purposes of paying reduced fees under § 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled

**"Composition and Methods Utilizing Stable Reporter Cell Lines for
Detection of Pathway-Specific Signal Transduction"**

by inventor(s) Chao-Feng, Zheng
described in

- ☒ the specification filed herewith
☐ Application Serial No. _____ filed _____
☐ Patent No. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern with regard to the above-identified invention.

If the rights held by the above identified small business concern are not exclusive, each individual concern or organization having rights in the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR § 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR § 1.9(d), or a nonprofit organization under 37 CFR § 1.9(e).

Ronni Sherman
NAME OF PERSON SIGNING

Executive Vice President & General Counsel
TITLE IN ORGANIZATION

STRATAGENE

11011 North Torrey Pines Road, La Jolla, CA 92037
COMPANY

SIGNATURE

DATE

FIELD OF THE INVENTION

The invention relates in general to the measurement of the activation status of signal transduction pathways and/or components of these pathways in cells. More specifically, the invention relates to engineered cell lines and methods using such cell lines for the measurement of the activation/inhibition of signal transduction pathways converging at transcriptional activators in cells.

BACKGROUND OF THE INVENTION

A multicellular organism is composed of many types of cells performing specialized functions. Cells communicate with each other for the organism to function as a whole. They do so at many levels and by various mechanisms. Cell identity is determined by the proteins that are synthesized within the cell. Therefore, regulation of gene expression, especially transcription, is the key mechanism of controlling cell growth and differentiation. To control transcription in response to extracellular stimuli originating from other cells or the surrounding environment, signals from outside the cell are transmitted to the transcription machinery inside the nucleus via a variety of signaling molecules including receptors, protein kinases and phosphatases and adapters, which form networks known as signal transduction pathways, or STPs (Boulikas, 1995, Crit. Rev. Euk. Gene Exp. 5: 1-77; Hunter et al., 1992, Cell 70: 357-387; Karin and Hunter, 1995, Curr. Biol. 5, 747-757; Treisman, 1996, Current Opinion in Cell Biol. 8: 205-215). These signaling molecules are also of interest to medical science and to the pharmaceutical industry because malfunctions of these molecular pathways are the cause of many human diseases, including cancers, genetic disorders and immune diseases. Therefore, many of the intracellular signaling molecules are targets for drug

intervention and targets for drug screening.

Among the best characterized STPs are the cAMP-dependent protein kinase (PKA) pathway and the mitogen-activated protein kinase (MAPK) pathways mediating signals from growth factors (e.g. EGF and NGF) and cellular stress such as heat, UV, oxidative stresses and protein synthesis inhibitors (Fig. 1). These signaling pathways receive diverse upstream signals and cause distinct downstream changes. One common feature of these pathways is that they all rely on the nuclear translocation of an activated protein kinase (e.g. MAPK or PKA) to transmit signals from outside the cell to the transcription machinery in the cell nucleus (Fig. 1). When activated by upstream signals, signal transduction kinases specific to a given pathway (e.g., MAPK or PKA) translocate into the nucleus and phosphorylate critical residues thereby activating the transactivating activity of a specific transcription factor(s), and thus converting an extracellular signal into a specific transcriptional response in the nucleus (Fig 1). The activation status of the transcription factors therefore reflects the activation status of the respective kinases and upstream signaling molecules along the pathway.

Since the discovery of the first member of the MAPK family (Ray and Sturgill, 1987, Proc. Natl. Acad. Sci. USA 84: 1502-1506), more than one hundred MAPK family members have been cloned (Kultz, 1998, J. Mol. Evol. 46: 571-588). These signaling pathways all use a three-component protein kinase cascade consisting of MAPK/MAPK kinase/MAPK kinase kinase but receive diverse upstream signals and cause distinct downstream changes. In the budding yeast *S. cerevisiae*, at least five distinct MAP kinase pathways have been identified to function in mating (Fus3/Kss1), cell wall biosynthesis (Mpk1), osmosensing (Hog1), sporulation (SmK1) and pseudohyphal development and invasive growth pathway (Levin and Errede, 1995, Current Opin. Cell Biol. 7: 197-202; Waskiewicz

and Cooper, 1995, Curr. Opin. Cell. Biol. 7, 798-805). In mammalian cells, over 12 MAPKs have been cloned and characterized (Kultz, 1998, *supra*; Waskiewicz, 1995, *supra*). One group of MAPKs is the classical MAPKs including ERK1 and ERK2, abbreviated from extracellular signal-regulated kinases (Ray and Sturgill, 1987, *supra*; Cobb and Coldsmith, 1995, J. Biol. Chem. 270: 14843-14846; Boulton et al., 1991, Cell 48: 663-675; Enslen et al., 1996, Proc. Natl. Acad. Sci. USA 93: 10803-10808; Marais et al., 1993, Cell 73: 381-393; Lin et al., 1995, Science 268: 286-289). They were first identified in insulin treated adipocytes and mainly relay signals from growth factors and the cancer promoting agent PMA.

The CREB transcription factor is activated by PKA, which is in turn activated by the second messenger cyclic AMP (cAMP) (Flint and Jones, 1991, Oncogene 6: 2019-1026). CREB is also activated by the growth factor-regulated kinase RSK2 (Xing et al., 1996, Science 273: 959-963). CREB activity thus reflects the activation status of those factors upstream of CREB in the activation pathway.

Also among the transcription factors activated by MAPK pathways is CHOP/GADD153, which will be referred to herein as CHOP (Wang and Ron, 1996, Science 272: 1347-1349). CHOP is activated by p38 MAPK, which is in turn activated by the MAPKK MEK3. CHOP activity thus reflects the activation of those factors upstream of CHOP in the activation pathway.

One group of MAPKs in mammalian cells is the stress-activated protein kinases (SAPKs), including c-Jun N-terminal kinase (JNK). Each SAPK has multiple isoforms and splicing variants (Kultz, 1998, *supra*; Waskiewicz and Cooper, 1995, *supra*; Derijard et al., 1994, Cell 76: 1025-1037; Kyriakis et al., 1994, Nature 369: 156-160; Minden et al., 1995, Cell 81: 1147-1157; Lin et al., 1995, Science 268: 286-289; Smeal et al., 1994, EMBO J. 13: 6006-6010; Lee et al., 1996, Mol. Cell. Biol.

16: 4312-4326). SAPKs, as the name implies, relay signals from diverse cellular stresses, such as UV exposure, heat shock, osmotic and oxidative stress, as well as from cytokines such as TNF- α and chemical agents such as cycloheximide.

Transcriptional activator proteins have been found to be modular in nature, very often comprised of linked domains that retain their respective functions when separated from the remainder of the protein. This modular nature of transcription factors was originally demonstrated in the yeast GAL4 transactivator, and has subsequently been found in a wide variety of transcription factors wherein the activation domain (AD) and DNA binding domain (DBD) may be structurally and functionally separated (Ma and Ptashne, 1987, *Cell* 48: 847-853). An important consequence of the modular nature of transcription factors is that the isolated functional domains may confer the specific DNA binding or transactivating activity of a given transcription factor upon an unrelated fusion partner.

Fusion transactivators consisting of the DNA-binding domain of yeast GAL4 protein (amino acid residues 1-92 or 1-147; Sadowski and Ptashne, 1989, *Nucl. Acids Res.* 17: 7539) or *E. coli* LexA (residues 1-87; Thliveris and Mount, 1992, *Proc. Natl. Acad. Sci. USA* 89: 4500-4504) and the activation domains of transcription activators from higher eukaryotes have been used in the literature as sensors for specific pathways in transient transfection assays (Fig. 2; Xu et al., 1997, *Strategies* 10: 1-3; Xu et al., 1997, *Strategies* 10: 79-80; Xu et al., 1997, *Strategies* 10: 81-83; Sanchez et al., 1998, *Strategies* 11: 52-53; Baichwal and Tjian, 1990, *Cell*, 63: 815-826; Enslen et al., 1996, *Proc. Natl. Acad. Sci. USA*. 93: 10803-10808; Hill et al., 1993, *Cell*, 73: 395-406; Lin et al, 1995, *Science*, 268: 286-289; Livingstone et al, 1995, *EMBO J.*, 14: 1785-1797; Marais et al., 1993, *Cell* 73: 381-393; Minden, et al., 1995, *Cell*, 81: 1147-1157; Price et al., 1995, *EMBO J.*, 14:

measurement of the biological activities of steroid hormones (Jausons-Loffreda et al., 1994, J. Biolumin. Chemilumin. 9: 217-221; Braselmann et al., 1993, Proc. Natl. Acad. Sci. USA. 90: 1657-1661; Louvion et al., 1993, Gene 131: 129-134; Dang et al., 1991, Mol. Cell. Biol. 11, 945-962). The steroid hormone studies investigated the single component steroid hormone receptor pathway, in which the receptor itself translocates to the nucleus and transactivates gene expression after binding the hormone. Fusion proteins of this kind have not been used, however, to establish stable reporter systems responsive to multicomponent signal transduction cascade pathways such as the MAPK pathway. There is a need in the art for pathway-specific signal transduction assay systems that do not require transfection, whether transient or otherwise.

[illegible]

In preferred embodiments, the reporter gene is selected from the group consisting of luciferase, β -galactosidase, chloramphenicol acetyltransferase, secreted alkaline phosphatase and green fluorescent protein or other easily assayable reporter activity. More preferably, the reporter gene is luciferase. In other preferred embodiments, the recognition sequence for a sequence-specific DNA-binding domain is that sequence recognized by one of the group consisting of GAL4 and LexA.

The invention also encompasses a method of assaying for the activity of a signal transduction pathway in a mammalian cell, the method comprising the steps of: detecting in a signal transduction pathway-specific reporter cell line expression of a reporter gene, wherein the reporter cell line

modulator and in the absence of the candidate modulator is indicative of modulatory activity of the candidate modulator on the pathway.

In these methods, it is preferred that the method further comprises, during the contacting step, providing an activator signal, the signal activating the conditionally active transactivation domain. The methods also may comprise adding an activator compound to the culture medium of the reporter cell line.

In other preferred embodiments of these methods, the DNA binding domain is selected from the group consisting of the DNA binding domains of GAL4 and LexA.

Other preferred embodiments include wherein the mammalian cell is human, such as a HeLa cell line.

The invention also encompasses a method of assaying for the activation of a conditionally active transactivation domain in a mammalian cell, the method comprising the steps of: detecting in a stable reporter cell line that is subjected to conditions which permit activation of the conditionally active transactivation domain the expression of a reporter gene, the reporter cell line comprising a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, the fusion protein comprising a sequence-specific DNA binding domain, wherein the DNA binding domain specifically binds the recognition sequence, and a conditionally active transactivation domain, wherein activation of the conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, wherein binding of the fusion protein to the recognition sequence results in transactivation of the reporter gene when the transactivation domain fused to the DNA binding domain is activated; and wherein expression of

the reporter gene is indicative of the activity of the conditionally active transactivating protein.

The invention also encompasses a method of screening for a modulator of the activity of a conditionally active transactivation domain in a mammalian cell, the method comprising the steps of: (a) contacting a stable reporter cell line with a candidate modulator under conditions sufficient to permit activation of the conditionally active transactivation domain, the reporter cell line comprising a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, the fusion protein comprising a sequence-specific DNA binding domain, wherein the DNA binding domain specifically binds the recognition sequence, and a conditionally active transactivation domain, wherein activation of the conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, wherein binding of the fusion protein to the recognition sequence results in transactivation of the reporter gene when the transactivation domain fused to the DNA binding domain is activated; and (b) detecting the expression of the reporter gene, wherein a difference in expression of the reporter gene in the presence of the candidate modulator and in the absence of the candidate modulator is indicative of modulatory activity of the candidate modulator on the conditionally active transactivating protein.

In preferred embodiments, the methods may further comprise, during the contacting step, providing an activator signal, the signal activating the conditionally active transactivation domain. In other preferred embodiments, the methods may comprise adding an activator compound to the culture medium of the reporter cell line.

It also may be preferred that the DNA binding domain is selected from the group consisting of the DNA binding domains of GAL4 and LexA.

Preferably, the mammalian cell is human, such as a HeLa cell line.

The invention also encompasses a kit comprising a cell line described herein and packaging therefor, and preferably where the kit also comprises a stably integrated recombinant nucleic acid construct comprising a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, the fusion protein comprising a sequence-specific DNA binding domain, wherein the DNA binding domain specifically binds the recognition sequence, and a conditionally active transactivation domain, wherein activation of the conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, and wherein binding of the fusion protein to the recognition sequence results in transactivation of the reporter gene when the transactivation domain fused to the DNA binding domain is activated.

Preferably, the kit further includes a nucleic acid expression construct encoding an upstream activator of the conditionally active transactivation domain.

The invention also encompasses a method of assaying for the interaction of two proteins in a mammalian cell, the method comprising the steps of: (I) detecting in a mammalian cell expression of a reporter gene, wherein the mammalian cell contains in its genome a stably integrated recombinant reporter gene construct comprising a reporter gene operably linked to one or more copies of a sequence-specific recognition site, and the mammalian cell comprises (a) a recombinant nucleic acid construct encoding a first protein fused to a DNA binding domain that specifically binds the sequence-specific recognition site; (b) a recombinant nucleic acid construct encoding a second protein fused to an activation domain of a transcriptional activator protein, wherein the activation domain transactivates expression of a gene to which it is bound; and II) detecting the expression of

the reporter gene, wherein the expression indicates interaction of the first protein and the second protein in the mammalian cell.

The invention also encompasses a method of screening for a modulator of the interaction of two proteins known to interact in a mammalian cell, the method comprising the steps of: (I) contacting a mammalian cell with a candidate modulator, the mammalian cell containing in its genome a stably integrated reporter gene construct which comprises a reporter gene operably linked to one or more copies of a sequence-specific recognition site, and the mammalian cell also comprises (a) a nucleic acid construct encoding a first protein of an interaction pair fused to a DNA binding domain which specifically binds the sequence-specific recognition site; and (b) a nucleic acid construct encoding a second protein of an interaction pair fused to an activation domain of a transcriptional activator protein, wherein the activation domain transactivates expression of a gene to which it is bound; II) detecting the expression of the reporter gene, wherein a difference in reporter gene expression in the presence and absence of the candidate modulator is indicative of modulation by the candidate modulator of the interaction of the first and second proteins in the mammalian cell.

Preferably, the DNA binding protein or DNA binding domain thereof is selected from the group consisting of GAL4 and LexA.

Preferably, the cell line is a mammalian cell, more preferably a human cell line, and most preferably a cell line such as HeLa.

As used herein, the term "parent cell line" refers to a cell line that is transfected to generate a reporter cell line or a pathway-specific reporter cell line according to the invention.

A parent cell line must be eukaryotic, and is preferably a mammalian cell line, and more preferably a human cell line, and does not contain a recombinant DNA containing the activation domain fused

to the reporter gene.

As used herein, a “reporter cell line” is a cell line that carries a stably integrated reporter gene operably linked to a recognition sequence for the DNA binding domain of a sequence-specific DNA binding protein.

As used herein, the term “pathway-specific reporter cell line” refers to a reporter cell line in which the activity of the reporter gene reflects the activity of signal transduction pathways converging on a particular conditionally active transactivating protein or protein domain. A pathway-specific reporter cell line carries, in addition to a reporter construct, a conditionally active fusion transactivator protein construct that activates expression of the reporter in response to activation of a particular signal transduction pathway.

As used herein, the term “stably integrated” refers to incorporation of a nucleic acid construct into the genome of a host cell such that it is replicated when the genome is replicated and is passed onto the progeny cells upon cell division for at least two cell divisions and preferably for at least ten, more preferably for at least twenty to thirty cell divisions. As referred to herein, a stably integrated nucleic acid construct encompasses the integration of a single copy or of two, three, or up to five, ten, twenty or even one hundred or more copies of the nucleic acid construct. Stable integration over a number of cell divisions may be assessed by determining the presence of one or more copies of the construct in a given cell preparation using, for example, PCR amplification of a region of the genome containing the construct.

As used herein, the term “reporter gene” refers to a gene sequence encoding a product that is detectable when expressed by a host cell, the expression of which product is under the control of heterologous regulatory sequences conferring responsiveness of the reporter gene to the activation

of a particular regulatory pathway. In order to be useful as a reporter gene, the product, which may be an RNA transcript or a protein or protein activity, including but not limited to an enzyme or enzyme activity, should not be endogenous to the host cell, or at least should not be detectable in the host cell in an amount that renders detection of the exogenous gene product over the endogenous product impossible. Non-limiting examples of reporter genes useful in the invention include luciferase (from firefly or other species), chloramphenicol acetyltransferase, β -galactosidase and green fluorescent protein. In order to be useful according to the invention, the background expression of a reporter gene (i.e., the detectable expression of the reporter in the absence of a signal that activates the regulatory pathway to which the reporter is responsive) must be low. Reporter gene background may be the to be low if an induction of 10-fold, preferably 20 fold, 30 fold, or up to 50 to 100 fold or more is detectable within the linear range of the detection assay.

As used herein, the term "sequence-specific DNA binding protein" refers to a protein that recognizes and binds a specific DNA sequence. The sequence bound by a sequence-specific DNA binding protein may be an invariant arrangement of contiguous nucleotide residues (e.g., GGATCC) or it may be a conserved sequence motif in which individual residues may vary and still allow recognition and binding by the sequence-specific DNA binding protein (e.g., GGPuPyCC, wherein Pu and Py are purine and pyrimidine, respectively). Binding of the protein to its specific sequence may be assessed via any conventional protein:nucleic acid binding methods, including but not limited to electrophoretic gel analysis of a given protein:nucleic acid construct.

As used herein, the term "recognition sequence" or "recognition sequence for a sequence-specific DNA binding protein" or "sequence-specific recognition site" refers to the particular sequence or sequence motif of nucleic acid residues recognized and bound by a sequence-specific

DNA binding protein.

As used herein, the term “DNA binding domain” refers to a portion of a sequence-specific DNA binding protein that binds to the recognition sequence on DNA. The term “DNA binding domain” is meant to encompass a complete sequence-specific DNA-binding protein as well as the particular portion or domain thereof that is sufficient to permit or mediate DNA binding. It should be understood that a DNA binding domain according to the invention has the ability when separated from the context of the whole sequence-specific DNA binding protein to bind DNA in a sequence-specific manner, and further, that it can confer this DNA binding specificity upon another protein or portion of a protein when fused to it. It should be further understood that a DNA binding domain according to the invention does not transactivate gene expression.

As used herein, the term “fusion protein” refers to a recombinant protein comprising two or more proteins, or domains or portions of two or more proteins, linked together in a manner not occurring in nature.

As used herein, the terms “transcriptional activator protein”, “transactivating protein”, or “transactivation domain” refer to a protein or domain of a protein which can contribute to an increase in the transcription of a gene through interactions with the enzymes and factors that assemble at the promoter of a gene to form a functional transcription complex. A transactivating protein or transactivation domain may exist in an active form, capable of effecting an increase in transcription, or in an inactive form requiring activation before effecting an increase in transcription; a transactivating protein or transactivation domain of this type is referred to herein as “conditionally active”. It should be understood that a transactivating protein or transactivation domain can confer transactivating properties upon another protein or protein domain when expressed as a fusion with,

of hypoxia or oxidative stress, such treatment resulting in the activation of a signal transduction pathway or portion of a pathway in a cell.

As used herein, the term “activator compound” refers to a compound or chemical agent that can serve to generate an activator signal.

As used herein, the term “upstream activator” refers to a protein or treatment that activates a signal transduction pathway at or before the pathway step involving a conditionally active transactivation domain being used to drive expression of a reporter gene in a pathway-specific reporter cell line of the invention.

As used herein, the term “interaction pair” refers to two proteins with the capacity to physically interact with each other to form a complex. An interaction pair may comprise two proteins known to interact with each other. A “potential interaction pair” may comprise two known proteins being tested for the ability to interact, one known protein and one unknown protein being tested for the ability to interact, or even two unknown proteins being tested for the ability to interact.

The present invention is useful for obtaining consistent assay results in signal transduction assays, and employs cell lines to analyze regulation of particular signal transduction pathways. The invention also is useful for assessing the influence or function of gene products in particular signal transduction pathways, screening for candidate modulators of particular signal transduction pathways, and analyzing protein:protein interactions in mammalian cells.

Further features and advantages of the invention will become more fully apparent in the following description of the embodiments and drawings thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic of signal transduction pathways in mammalian cells.

As shown in the figure by solid arrows, MAPKs are phosphorylated and activated by MAPKKs directly, and they can then directly phosphorylate and activate downstream transcriptional activators. There may be many steps from the cell surface or other part of the cell to the activation of MAPKKS or other downstream activators.

Figure 2. Monitoring Pathway-Specific Signal Transduction..

A schematic of a pathway-specific signal transduction monitoring system is shown. A pathway-specific fusion transactivator plasmid and a reporter plasmid are cotransfected, optionally along with a plasmid encoding a gene of interest, into a eukaryotic cell. The fusion transactivator may then bind a recognition element linked to the reporter, and, depending on the activation status of the fused transactivation domain, may activate transcription of the reporter. The (positive or negative) effect of a gene of interest on the functions of the specific pathway may be determined by changes or differences in the expression of the reporter.

Figure 3. Selection procedure for selection of stable reporter cell lines.

A schematic of the selection of stable reporter cell lines is shown. A reporter construct with a linked DNA-binding domain element(s) is stably transfected into a chosen cell line, followed by screening for clones with low background reporter activity and a strong response to activators that bind the DNA-binding domain recognition element. The singly transfected stable reporter cell line is then stably transfected with a pathway-specific fusion transactivator plasmid, followed by screening for clones with a strong response to pathway-specific upstream activator(s)

to identify pathway specific stable reporter cell lines.

Figure 4. Schematic of pFR-Luc reporter plasmid.

A schematic diagram of the pFR-Luc reporter vector is shown. Five copies of the GAL4 DNA-binding domain recognition sequence (underlined) are linked to a minimal promoter containing a TATA element upstream of the initiator ATG initiator codon of firefly luciferase coding sequences.

Figure 5. pFA-CMV plasmid.

A schematic diagram of the plasmid pFA-CMV, used as the base vector for the fusion transactivator plasmids according to the invention is shown. The vector fuses the GAL4 DNA-binding domain (amino acids 1-147) to the selected fusion transactivation domain via the shown multiple cloning site. Expression of the resulting fusion transactivator protein is driven by the strong CMV promoter.

Figure 6. Initial screening of RCLH-Luc clones.

Cells ($\sim 5 \times 10^4$ per well) from various cells transfected with pFR-Luc (X axis), were clones of HeLa cells cotransfected with either 10ng/well of control plasmid (pBK-CMV, open bars) or 10 ng/well of pFC-MEKK (solid bars) together with 2 ng/well pFA2-cJun. Luciferase activity in the cells was determined forty eight hours after transfection as described in the Description. Clones with the highest luciferase activity were subjected to further characterization.

Figure 7. Activation of GAL4:Elk1 by MEK1.

Various amounts of pFC-MEK1 (solid squares) or pCMV-Script (open squares) as shown on the X axis were transfected into RCLH-Elk1 cells (5×10^4). DNA amounts of all the samples were kept constant with different amounts of an unrelated plasmid (pBluescript).

Figure 8. Activation of GAL4:Elk1 by extracellular stimuli.

RCLH-Elk1 cells (3×10^5) were treated with the listed stimuli for 5 hours prior to cell lysis and luciferase activity assays.

Figure 9. Effect of a gene product on pathways converging on Elk1.

The figure shows the results of transient transfection assays examining the effect of a gene product, either a constitutively active MEK (CA-MEK) or a dominant negative MEK (DN-MEK) on the expression of reporter in RCLH-Elk1 cells.

Figure 10. Screening for activators or inhibitors of a signal transduction pathway (drug screening).

The figure shows the results of treatment of RCLH-Elk1 cells with medium alone (DMEM), dimethylsulfoxide (DMSO), anisomycin, forskolin and 3-isobutyl-1-methylxanthine (IBMX), EGF, phorbol ester (PMA) or EGF and PMA.

Figure 11. Initial screening of RCLH-CREB clones.

Cells ($\sim 5 \times 10^4$ per well) from various clones of RCLH-CREB as labeled on the X-axis were transfected with either 10 ng/well of control plasmid (pBK-CMV, open bars) or 10 ng/well of pFC-PKA (solid bars). Luciferase activity in the cells was determined forty-eight hours after transfection.

Figure 12. Activation of GAL4-CREB by PKA in RCLH-CREB cells.

Various amounts of pFC-PKA (solid diamonds) or pCMVScript (open diamonds) as shown on the X-axis were transfected into RCLH-CREB cells ($\sim 3 \times 10^5$). DNA amounts in all samples were normalized with pBluescript.

Figure 13. Activation of GAL4-CREB by extracellular stimuli in RCLH-CREB cells.

RCLH-CREB cells (clone #1-8) were treated with the following stimuli for 5 hours prior to cell lysis and luciferase assays: DMSO (dimethyl sulfoxide, solvent control); AC Toxin (adenylate cyclase toxin, 10 and 50 ug/ml); and PMA (phorbol-12-myristate-13-acetate) plus Forskolin (10, 50 and 100 uM).

Figure 14. Initial screening of RCLH-CHOP clones.

Cells ($\sim 3 \times 10^5$ per well) from various RCLH-CHOP cell clones were transfected with varying amounts of pFC-MEK3 activator plasmid. Luciferase activity in the cells was determined forty eight hours after transfection.

Figure 15. Activation of luciferase expression by MEK3 in RCLH-CHOP cells.

RCLH-CHOP clone #1-4 cells ($\sim 3 \times 10^5$ per well) were transfected with varying amounts of pCMVScript (open squares) or pFC-MEK3 (solid squares). DNA amounts of all samples were normalized with an unrelated plasmid (pBluescript). Luciferase activity in the cells was determined forty eight hours after the transfection. Luciferase activity from the zero point of pCMVScript (open

square on the Y axis is an anomalous data point, as it was not observed in other experiments.

Figure 16. Stability of RCLH-CHOP cell lines.

RCLH-CHOP clone #1-4 cells ($\sim 3 \times 10^5$ per well) were transfected with various amounts of pFC-MEK3 as indicated on the X axis. Luciferase activity in the cells was measured forty-eight hours after transfection. The cells have either been continuously cultured (blank bars), stored at -80°C (shaded bars) or stored in liquid nitrogen (solid bars) for a period of over three months.

Figure 17. Activation of luciferase expression by MEKK in RCLH-cJun cells.

RCLH-cJun clone #1-4 cells ($\sim 3 \times 10^5$ per well) were transfected with various amounts of pFC-MEKK (solid squares). DNA quantities in all samples were normalized with an unrelated plasmid (pBluescript). Luciferase activity in the cells was determined forty-eight hours after transfection.

Figure 18. Phenotypic stability of RCLH-cJun cells.

RCLH-cJun clone #1-4 cells ($\sim 3 \times 10^5$ per well) were transfected with various amounts of pFC-MEKK as indicated in the X-axis. Luciferase activity in the cells was determined 48 hours after transfection. The cells have either been continuously cultured (blank bars) or stored at -80°C (shaded bars) or in liquid nitrogen (filled bars) for a period of over 3 months.

Figure 19. Detection of Protein:Protein Interaction with the RCLH-Luc cell line.

RCLH-Luc Cells ($\sim 3 \times 10^5$ per well) were transfected with either 0.25 (open bars) or 1.0 ug (solid bars) of the plasmids or plasmid combinations as labeled on the X-axis. Luciferase activity in the cells was determined forty eight hours after transfection as described in Materials and Methods. BD: mammalian expression plasmid for GAL4 DBDd; p53: fusion plasmid of GAL4 DBD and p53 protein; AD: expression vector for GAL4 activation domain; SV 40: fusion plasmid of GAL4 activation domain and SV 40 large T antigen.

DETAILED DESCRIPTION OF THE INVENTION

The present invention addresses the need in the art for assay systems designed to provide consistent results in analyses of signal transduction pathways. The cell lines and assay systems disclosed herein are well suited for use in pathway-specific signal transduction assays to screen for candidate modulators of those signal transduction pathways, as well as for the study of transcriptional regulatory mechanisms in mammalian cells.

The invention relates to a cell line comprising a stably integrated recombinant nucleic acid construct comprising a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein that responds to the activation of a particular signal transduction pathway.

The invention also relates to a cell line comprising a stably integrated recombinant nucleic acid construct comprising a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein, and further comprising a stably integrated nucleic acid construct comprising a sequence encoding a transactivator fusion protein, such fusion protein comprising a DNA binding domain, wherein the DNA binding domain specifically binds the recognition sequence

for a DNA binding protein operably linked to the reporter, and a conditionally active transactivation domain, wherein binding of the fusion protein to the recognition sequence results in transactivation of the reporter gene when the conditionally active transactivation domain fused to the DNA binding domain is activated.

In one embodiment of the invention, a cell line is generated which carries one or more stably integrated copies of a reporter gene operably linked to at least one copy, preferably at least two copies or more, up to as many as five copies or more of the recognition sequence for the DNA binding domain of a sequence-specific DNA binding protein. A cell line of this type will be referred to herein as a reporter cell line.

As copies of a reporter plasmid (e.g., the luciferase reporter plasmid pFR-Luc) and a fusion transactivator plasmid are integrated into the chromosomes and become part of the genome in a reporter cell line (e.g., RCLH-Luc), the expression of reporter from them is subjected to many regulatory mechanisms unique to the natural state of genes on a chromosome. Stable integration of nucleic acids encoding reporters and transactivator fusion proteins improves the consistency and simplicity of assays for particular signal transduction pathways beyond that of transient transfection systems and facilitates high-throughput applications. Using the resulting single or double stably transfected reporter cells, the effects of extracellular stimuli on signaling pathways converging at a particular transactivating protein can be directly assessed by reporter assays without the need for transfection. These cell lines can also be used to study transcription mechanisms, and may be adapted for high-throughput drug screenings and panel assays. Therefore, the cell lines are useful tools for studying the regulation of the transcription machinery in vivo and to probe the roles of chromosomal structure in gene expression control.

Reporter Cell Lines Useful According to the Invention

A reporter cell line may be established from any desired eukaryotic cell line, preferably a mammalian cell line and not a yeast cell. A human cell line is of particular interest according to the invention. Methods of creating cell lines are well known in the art, and methods of creating stably transfected cell lines bearing at least one or more integrated constructs are also known in the art. Detailed descriptions of the various aspects of reporter cell lines according to the invention are presented below.

A. Parent Cell Line.

In order to be useful as a parent cell line according to the invention, the cell line should contain the protein kinases that activate the fusion transactivation protein. In other words, the pathway one wishes to monitor should be functional in the chosen cell type. Cell lines vary in signaling proteins and other properties. The endogenous protein kinases and transcriptional activator activities in the cell line will determine the background, and hence, the sensitivity of the assay. Further, the parental cell line must clearly be both transiently and stably transfectable, as readily determined by one skilled in the art. See Tables I and II for examples of experimental approaches to determine the activity of a pathway and reporters in a given cell line.

The examples described herein refer to the use of HeLa cells (ATCC #CCL-2) as the parental cell line. This should in no way be construed as limiting the invention to the use of HeLa cells.

B. Reporter Genes and Reporter Gene Expression Assays.

Reporter genes and assays for the expression of the reporter genes are widely known in the art. In order to be useful according to the invention, a reporter gene must have the following

properties: i) its product (either nucleic acid transcript, protein, or protein activity) should be readily detectable; and ii) a reporter and detection system should preferably be amenable to high throughput applications. Another property to consider when choosing a reporter system is the stability of the product (Thompson et al., 1993, Gene 103: 171-177). Depending upon the exact use, one may wish to use a reporter whose measurable product has relatively high stability in the cell. Use of a stable reporter product will allow sensitive detection of pathway activity in terms of on versus off status; an example of a relatively stable reporter is the chloramphenicol acetyltransferase (CAT) gene product. However, in cases in which one wishes to detect changes such as a decrease in reporter gene activity, a relatively less stable reporter gene product or activity would be more desirable since it would more rapidly read out decreases in expression; an example of a relatively less stable reporter gene product is firefly luciferase. Reporter genes of use in the invention include, but are not limited to those encoding firefly luciferase (deWet et al., 1987, Mol. Cell. Biol. 7: 725-737) or luciferase genes from other species), β -galactosidase, CAT and green fluorescent protein (GFP). Constructs encoding such reporter molecules are widely commercially available or may be obtained from sources such as ATCC.

In order to be useful for pathway-specific signal transduction assays, a reporter cell line (or a cell line bearing a reporter and a DNA binding domain:conditionally active transactivation domain fusion protein, referred to herein as pathway-specific reporter cell lines) must exhibit low background reporter activity in the absence of an activating signal. The definition of a low background level of reporter activity is clearly relative. What is important is that the level of reporter activity is sufficiently low that increases of one or more orders of magnitude may be detectable within the assay parameters when reporter gene activity is induced. Generally, one should avoid

background levels wherein a tenfold or higher level of subsequent induction would saturate the assay (i.e., result in a non-linear signal response). When comparing a number of single or double stably transfected cell lines, it will be clear to one of skill in the art which one has the lowest background expression of reporter in the absence of an activating signal.

A strong reporter response upon treatment to activate the activator fusion will also be clear to one of skill in the art, particularly when comparing a number of singly or doubly transfected cell lines. It should be noted that while low background and strong response to activating signals are the desired ideals, the cell line with the lowest background will not necessarily be the line with the strongest response to activators. It is within the ability of one of ordinary skill in the art to choose a reporter cell line, from among a number of given reporter cell lines, which has the optimal balance of low background and strong responsiveness to activator treatment.

Reporter gene expression may be assayed according to standard methods known in the art and appropriate for the chosen reporter gene.

While the invention is not intended to be limited to using a particular reporter, firefly luciferase is well suited for the methods of the invention. In the assays shown in subsequent Examples, cell lysates were prepared and tested for luciferase activity using the Luciferase Assay Kit (Stratagene) according to manufacturer's specifications (Further information regarding luciferase assay methods may be found in Brasier, et al., 1992, Methods Enzymol. 216: 386-97). Typically, cells were lysed with 100 ul of lysis buffer (40 mM Tricine (pH 7.8), 50 mM NaCl, 2 mM EDTA, 1 mM MgSO₄, 5 mM Dithiothreitol (DTT) and 1% Triton X-100) and 20 ul were used per assay. Lysis may alternatively be performed by freeze-thaw, although this method is known to reduce luciferase activity by as much as 50% per cycle. Cell lysate was mixed with 100 ul of luciferase

example, nuclear translocation of NFAT proteins after dephosphorylation by calcineurin) or proteolysis (for example, cleavage of inactive precursor such as NF- κ B p105 to generate an active p50 NF- κ B p50 subunit) also exist. It is important that the DNA-binding domain-transactivator fusion protein does not activate transcription in the absence of a factor or signal which activates the conditionally active transactivator domain. This conditional activity makes the DNA-binding domain-activator fusion protein particularly useful according to the invention. A DNA-binding domain-transactivator fusion which is only active when stimulated by signaling proteins in the specific pathway being examined will reliably read out the activity of the particular signal transduction pathway by stimulation of reporter gene activity.

To be chosen for use in the cell lines and methods of the invention, it is assumed that the conditionally active, pathway-specific transactivation domain is known to be conditionally active and pathway specific. A particular conditionally active transactivation domain may be identified within a conditionally active transactivator protein through systematic deletion mutagenesis as follows. A nucleic acid sequence encoding the entire transactivator protein or portions thereof may be fused in-frame with a sequence encoding a sequence-specific DNA-binding domain. A panel of systematic deletion mutants (generated according to any one of several methods known in the art) may then be tested for transactivation activity by cotransfecting them along with a reporter gene construct bearing linked copies of the DNA-binding domain recognition sequence in the presence of an activating signal for the conditionally active transactivating protein. The smallest portion of the conditionally active transactivator protein found to retain transactivating activity identifies the transactivation domain. One of skill in the art may determine by incubating transfected cells in the presence and absence of an activating signal (a chemical or a co-transfected upstream activator) for

the transactivation domain whether the identified transactivation domain retains its conditional activity. A conditionally active transactivator will induce reporter gene expression in this assay only in the presence of such a specific activator of the transactivation pathway.

It is preferred that the conditionally active transactivator fusion protein be constitutively expressed. In this way, differences in reporter activity accurately reflect differences in pathway activity, rather than differences in activator expression. Under some circumstances, however, it may be desirable to express the activator fusion from a tissue- or cell type-specific promoter, or from an inducible promoter. The use of a cell type-specific promoter may, for example, ensure higher expression of stably integrated copies of the activator fusion protein. For example, if liver-derived cells are to be used, one may prefer to use a liver-specific promoter, such as the albumin or alphafetoprotein promoter to drive high level expression of the fusion activator.

F. Transactivator Fusion Protein Expression Constructs.

The transactivator fusion protein sequence may be included in any one of a variety of expression vectors. It is necessary that the vector chosen is capable of chromosomal integration; i.e., the chosen vector should not carry signals promoting episomal maintenance. Any plasmid or vector meeting this condition and being replicable in the chosen host cell without harming the viability of the host cell is acceptable according to the invention.

The conditionally active transactivator fusion protein construct may include sequence encoding a leader sequence that may be cleaved by the host cell to generate the mature form of the polypeptide. In addition, the encoded transactivator fusion protein may contain a prosequence, which is the mature protein plus additional amino-terminal amino acid residues. A mature protein having a prosequence is a proprotein and may in some cases be an inactive form of the protein. Once the

prosequence is cleaved an active mature protein remains. Thus, for example, the transactivator fusion protein of the invention may be encoded by a nucleic acid construct encoding a mature protein, a protein having a prosequence or a protein having both a presequence (leader sequence) and a prosequence.

As hereinabove indicated, the transactivator fusion protein DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into appropriate restriction endonuclease sites by procedures known in the art. Such sites may be existing sites or sites created or modified according to methods known in the art. Specific information on methods involved in cloning and mutagenesis may be found, for example, in Ausubel et al. (Ausubel et al., 1988, *Current Protocols in Molecular Biology*, (John Wiley and Sons, Inc.)) and in Sambrook et al. (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, 1989, (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.)).

The DNA sequence in the transactivator fusion protein expression vector is operatively linked to an appropriate, preferably eukaryotic (or at least functional in eukaryotic cells) expression control sequence (promoter) to direct mRNA synthesis. An appropriate control sequence is one which directs expression of the linked construct in the chosen cell type. Examples of such promoters include but are not limited to viral control sequences (e.g., retroviral LTRs, SV40 promoter, Adenoviral promoters, CMV promoter, HSV promoter, etc.) and promoters for eukaryotic cellular genes such as the mouse metallothionein promoter, housekeeping gene promoters such as GAPDH, or even tissue-specific promoters such as the albumin or immunoglobulin promoters.

Transcription of a DNA construct encoding the transactivator fusion protein may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA,

usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin (bp 100 to 270), a cytomegalovirus early promoter enhancer, a polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The transactivator fusion protein expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression. The expression vector may additionally be regulated in an inducible manner, such as by addition (or removal) of a chemical regulator (e.g., IPTG, tetracycline), by co-expression of an inducer or repressor (e.g., *lac* repressor, ecdysone receptor) or by other means known in the art.

For more information regarding expression vectors active in mammalian cells, see, for example, Kaufman, 1990, Meth. Enzymol. 185:487-511.

To facilitate the integration and selection for stable reporter gene integration, a hygromycin resistance expression cassette, excised from p3'SS (a vector for LacSwitch™ expression systems (Stratagene), GenBank Accession No. U42371), was inserted into the NdeI site of the pFR-Luc (Genbank Accession No. AF058756) luciferase reporter vector, to generate pFR-Luc-Hyg. pFR-Luc (and therefore pFR-Luc-Hyg) carries five copies of the GAL4 DNA-binding domain recognition sequence 5'-CGGAGTACTGTCCTCCG-3' upstream of a basic TATA element and the coding region for firefly luciferase (see Figure 4).

Plasmid DNAs used for transfection were purified from *E. coli* XL1-Blue cells (Stratagene) with Qiagen maxi prep kits or by CsCl banding. Clearly, one of skill in the art may prepare plasmid DNAs using other strains of *E. coli* or other bacteria as known in the art.

2. Preparation of genomic DNA

Genomic DNAs from cultured cells were prepared using Stratagene's DNA Extraction Kit (Cat#200600) according to the instruction manual using $\sim 6 \times 10^6$ cells. Alternatively, other methods known in the art may be applied in order to isolate genomic DNA from cultured cells (See, for example, Maniatis et al., 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, p. 280).

3. Cell Culture

HeLa cells were kept and cultured in DMEM, 1% L-Glutamine supplemented with antibiotic/antimycotic liquid (Gibco BRL) and 10% fetal bovine serum (Hyclone or Gemini), according to standard protocols. Other established cell lines (as available from various commercial sources, or, for example, from American Type Culture Collection, Manassas, VA) may be cultured according to the conditions published for such lines or as determined by one of skill in the art. Cell

lines for use in the invention may be derived from any eukaryote, preferably a mammal, and more preferably a human.

4. Transient Transfection

Transfections were performed in 24 well dishes using LipofectAMINE™ (Life Technologies) or LipoTaxi™ (Stratagene) following manufacturer's guidelines (other companies offering lipid-mediated transfection reagents include Bio-Rad Laboratories, CLONTECH, Glen Research, InVitrogen, JBL Scientific, MBI Fermentas, PanVera, Promega, Quantum Biotechnologies, Sigma-Aldrich, and Wako Chemicals USA). Briefly, cells were seeded at 5×10^4 cells per well and incubated overnight at 37°C. DNA lipid mixtures were prepared according to manufacturer's recommendations, incubated at room temperature for 20 - 40 minutes and overlaid onto the cells (0.25 ml per well). Equal volume of medium containing 1% fetal bovine serum was added to each well 5 hours after transfection. Medium was replaced 18 - 24 hours post transfection with fresh DMEM containing 0.5% fetal bovine serum. Cell lysates were collected 48 hours after transfection and assayed for luciferase activity.

Transient transfections may also be performed using the calcium phosphate precipitation, electroporation or DEAE dextran methods as known in the art (Ausubel et al., 1992, Short Protocols in Molecular Biology (John Wiley & Sons, NY), pp. 9-5 to 9-14).

5. Determination of stable integration of transfected constructs

Stable integration of transfected constructs may be determined using PCR and genomic cellular DNA (Mullis & Faloona, 1987, Methods Enzymol., 155:335). To ascertain reporter integration, PCR primers corresponding to the linked recognition sequence for the DNA-binding domain and the reporter coding region are appropriate. Amplification of a band of the expected size

following the selection protocol will verify the integration of one or more intact (that is, retaining the recognition sequence(s) and the reporter coding region) copies of the reporter construct. To assay for stable transfection with fusion transactivator constructs, one PCR primer should be specific for the fusion DNA-binding domain domain (e.g., GAL4, LexA, etc.) and the other should be specific for the fused transactivation domain.

H. Determining the Activation Status of a Transactivating Protein.

A stable reporter cell line stably transfected with a nucleic acid construct encoding a conditionally active transactivator fusion protein (that is, a pathway-specific reporter cell line) may be used to assay for the activation status of the conditionally active transactivation protein or activation domain thereof and/or the activation status of the signal transduction pathway of which the conditionally active transactivation protein or domain is a participating member. Such an assay would comprise the step of performing a reporter gene expression assay on such double stably transfected cells. The detected reporter gene activity reflects the activation status of the conditionally active transactivator, and thus reflects the activation status of the signal transduction pathway.

A double stably transfected, pathway-specific reporter cell line may also be used to screen for a candidate modulator of the selected signal transduction pathway. Such an assay comprises the steps of incubating such a double stably transfected pathway-specific reporter cell line in the presence or absence of a candidate modulator compound and detecting reporter gene activity. A change or difference (increase or decrease) in the level of reporter gene activity in the presence of a candidate modulator compound relative to the expression in the absence of the candidate modulator compound is indicative of a modulatory effect of such a candidate compound. Such an assay performed in the absence of a known activating signal (i.e., a signal that activates the conditionally

active transactivation domain on the fusion transactivator) may be used to screen for an activator of the signal transduction pathway. Alternatively, an assay performed in the presence of a known activating signal may be used to screen for either a further activating activity or for an inhibiting activity.

I. Activating Signals.

Activating signals may comprise chemical compounds known to specifically activate the specific signal transduction pathway being assayed (e.g., dibutyryl cAMP activation of the cAMP-dependent CREB pathway), or they may comprise treatments such as growth factor addition, thermal shock, induction of oxidative stress, or exposure to UV or other irradiation. Activating signals and treatments specific for particular signal transduction pathways are known in the art. In addition to activating signals or compounds known in the art, one may use an activating compound, identified by screening candidate compounds in a pathway-specific reporter cell line in the absence of a known activating signal, as described in the previous section.

Alternatively, an activating signal may comprise a treatment of the reporter cell line that induces the expression of an upstream activator of the signal transduction pathway being studied. Such an upstream activator may be, for example, a regulatory kinase acting upstream of the activator used as the fusion activator. In this instance, a nucleic acid construct encoding an upstream activator may be transfected, either transiently or stably (under control of an inducible promoter), into the reporter cell line. The expression of an upstream activator from a transiently-transfected activator construct or from a stable, inducible upstream activator construct will then induce the activity of the signal transduction pathway being studied. The upstream activator may be expressed in either its native form, or, alternatively, as a constitutively active mutant form. Inducible expression systems

are well known in the art; non-limiting examples include those induced by heat, the presence or absence of an antibiotic (e.g., tetracycline) or hormone (e.g., ecdysone), or the presence of activators such as IPTG (e.g., the LacSwitch™ system; Stratagene).

As another alternative, an activating signal may be provided in the form of a transfected, conditionally active upstream activator protein. In contrast to the situation in which *expression* of an upstream activator is induced, in this case the *activity* of the upstream activator is conditionally induced. Examples include temperature sensitive mutants of upstream activators, e.g., p53. A temperature shift results in a conformational change in the factor that activates or inactivates the upstream activator (see, for example, Milczarek et al., 1999, Carcinogenesis 20: 1043-8).

J. Investigation of Protein:Protein Interactions in Mammalian Cells Using Reporter Cell Lines of the Invention:

In addition to being used as a starter cell line for doubly transfected stable cell lines (see below), cell lines bearing a stably integrated reporter can serve as the reporter cell line for many other applications using GAL4 DNA-binding domain or other DNA binding domain fusion proteins. One such application is to test protein-protein interactions with mammalian two-hybrid vectors. In such a system, the two proteins or protein domains being tested for interaction (an interaction pair or potential interaction pair) are fused, respectively, to either member of a functional transactivation domain: DNA-binding domain pair. The first protein or protein domain may be fused, for example, to the GAL4 DNA-binding domain and the second protein or protein domain may be fused, for example, to the DNA-binding domain of GAL4 protein. If these two fusion proteins physically interact within the cell, via the first and second protein moieties, the GAL4 activation domain will be recruited to the reporter gene via the operably linked GAL4 DNA-binding domain recognition

sequence elements and thus activate reporter gene transcription. The first and second proteins or protein domains being tested for interaction may be known, or, alternatively, only one of the proteins or domains may be known and fused, for example to the GAL4 DNA binding domain. In such an instance, the second, or unknown protein or domain may be drawn from a library of sequences fused in-frame with, for example, the GAL4 activation domain. In this way, one may use the single stably transfected reporter cells to identify novel interaction partners for known proteins in mammalian cells.

It is also possible to adapt such an assay of protein:protein interactions for the identification of a modulator of a protein:protein interaction in a mammalian cell. To do so, one may perform the assay for a known protein:protein interaction pair in cells either in the presence or absence of a candidate modulator. A change (decrease or increase) in the expression of the reporter in the presence of the candidate modulator relative to the expression of the reporter in the absence of the candidate modulator would be indicative of a modulatory effect on the interaction by the candidate modulator.

K. Assay to screen for a modulator of signal transduction pathways:

An assay to screen for a modulator of a specific signal transduction pathway may comprise the following steps:

- 1) A reporter cell line stably transfected with a construct encoding a transactivator fusion protein responsive to signals in the specific signal transduction pathway being examined is treated with an activator of the specific signal transduction pathway in the presence and in the absence of a candidate modulator compound. The activator may be a known chemical inducer of the signal transduction pathway, a growth factor known to activate the signal transduction pathway, or an agent

or treatment that induces expression of an activator of the pathway. This includes expression of a candidate modulator protein from a library of candidate genes cloned into an expression vector that directs high expression in the chosen cell type.

2) Reporter activity is detected in the cells, with a change or difference (of 10% or more) in reporter activity in the presence of the candidate modulator relative to the absence of the candidate modulator being indicative of modulatory activity of the candidate modulator.

L. Examining the effect of a gene product on a signal transduction pathway.

Typical initial experiments for a *trans*-reporting system are outlined in Tables I and II. As

all assays are to be run in triplicate, eight samples will utilize four 6-well tissue culture dishes. Sample numbers are indicated in Column A. Column B indicates volumes of reporter plasmid to use. Column C indicates the amount of fusion *trans*-activator plasmid to be used in each sample. Column D indicates the amount of pFC2-DBD (negative control for the pFA plasmid to ensure the effects observed are not due to the GAL4 DNA binding domain; a similar control should be included for other DNA-binding domains) to be used in each sample. Column E indicates the appropriate volume of positive control upstream activator expression plasmid (specific to the pathway being examined) to be used. Column F indicates volumes of the experimental mammalian expression plasmid containing the gene of interest. Column G indicates amounts of the negative control for the plasmid used to express the gene of interest (i.e., plasmid without an inserted gene of interest). Column H indicates the amount of unrelated plasmid DNA containing no mammalian promoters or other elements to be used to keep the amount of DNA in each sample constant.

Table I outlines an approach to evaluating the effect of a gene product on a particular pathway using transient transfection of all system constituents. The stable pathway-specific reporter cells of the invention may also be used to evaluate the effect of a gene product on a particular pathway, with the advantage that the amount of reporter and fusion transactivator plasmids will be constant in all samples, thereby removing a major source of inaccuracy in this type of assay. In addition, the use of stable pathway-specific reporter cell lines avoids the potential for promoter interference that exists when multiple different expression/reporter plasmids are transiently transfected into a cell. That is, in the stable system, there should be little or no competition among transfected plasmids for transcription factors necessary for the expression of the transfected genes.

To examine the effect of a gene product on a particular activation pathway using pathway-

specific reporter cells, one may transfect a vector expressing the gene product or an empty vector into the cells and compare the activity of the reporter in both cases. Performed in the absence of an activating signal this method will assay for activators of the pathway. Performed in the presence of an activating signal (as described herein), the method will assay for both activators and inhibitors of the pathway. This approach is well suited for identifying a dominant negative or constitutively active regulatory factor mutant.

A gene of interest may be determined to influence the pathway if its expression results in a 10% or greater change in the level of reporter expression or activity after consideration of the relevant positive and negative controls.

TABLE 1

Sample Experiment to Study the Effects of a Gene Product

A	B	C	D	E	F	G	H
	#	Fusion <i>trans</i> - activator plasmid ^a	pFC2-DBD (negative control for pFA plasmid)	Positive control	Experimental plasmid with gene of interest	Experimental plasmid without insert	Plasmid DNA
	1.0 µg (µl)	50 ng (2 µl)	-	-	-	50 ng	950 ng
2 ^c	1.0 µg (µl)	50 ng (2 µl)	-	-	-	100 ng	900 ng
3 ^d	1.0 µg (µl)	50 ng (2 µl)	-	-	-	1000 ng	-
4 ^e	1.0 µg (µl)	50 ng (2 µl)	-	-	50 ng	-	950 ng
5 ^f	1.0 µg (µl)	50 ng (2 µl)	-	-	100 ng	-	900 ng
6 ^g	1.0 µg (µl)	50 ng (2 µl)	-	-	1000 ng	-	-
7 ^h	1.0 µg (µl)	50 ng (2 µl)	-	50 ng (2 µl)	-	-	950 ng
8 ⁱ	1.0 µg (µl)	-	-		100 ng	-	850 ng

^a This quantity may need to be optimized, usually within the range of 1-100 ng.

^b Sample 1 lacks the gene of interest and, therefore, controls for sample 4.

^c Sample 2 lacks the gene of interest and, therefore, controls for sample 5.

^d Sample 3 lacks the gene of interest and, therefore, controls for sample 6.

^e Sample 4 measures the effect of the gene product on the signal transduction pathway involved.

^f Sample 5 measures the effect of the gene product on the signal transduction pathway involved.

^g Sample 6 measures the effect of the gene product on the signal transduction pathway involved.

^h Sample 7 measures the efficacy of the assay for the cell line chosen.

ⁱ Sample 8 does not contain an activation domain and should show results similar to samples 1-3.

M. Examining the effect of an extracellular stimulus on particular signal transduction pathways.

The stable reporter cell line systems as described herein may also be used to study the effects of extracellular stimuli, such as growth factors, cellular stresses or drug candidates, on corresponding signal transduction pathways. Table II shows sample conditions for the assay of extracellular stimuli on a specific pathway using transient transfection assays. Cells are transfected with the fusion transactivator plasmid and then treated with the stimulus of interest. The same experiment may be performed without the need for transfection using the stable pathway-specific reporter cell lines of the invention. Reporter expression from the reporter plasmid indicates the activation of the fusion transactivator protein and, therefore, the presence and/or activity of the endogenous protein kinase specific to the activation pathway. Changes or differences in the expression of reporter in response to extracellular stimuli are therefore indicative of effects of those extracellular stimuli on the pathway.

TABLE II

Sample Experiment to Study the Effects of Extracellular Stimuli

#	pFR-Luc Plasmid (reporter plasmid)	Fusion <i>trans</i> - activator Plasmid ^a	pFC2-DBD (negative control)	Positive control	Extracellular stimuli
1 ^b	1.0 µg (µl)	-	50 ng (2 µl)	-	Serum (10%)
2 ^c	1.0 µg (µl)	50 ng (2 µl)	-	-	Serum (10%)
3 ^d	1.0 µg (µl)	-	50 ng (2 µl)	-	EGF (100 ng/ml)
4 ^e	1.0 µg (µl)	50 ng (2 µl)	-	-	EGF (100 ng/ml)
5 ^f	1.0 µg (µl)	-	50 ng (2 µl)	-	Medium
6 ^g	1.0 µg (µl)	50 ng (2 µl)	-	-	Medium
7 ^h	1.0 µg (µl)	50 ng (2 µl)	-	50 ng (2 µl)	-
8 ⁱ	1.0 µg (µl)	-	50 ng (2 µl)	-	-

- ^a This quantity may need to be optimized, usually within the range of 1-100 ng.
- ^b Sample 1 lacks the fusion *trans*-activator protein and, therefore, controls for sample.
- ^c Sample 2 measures the effect of fetal bovine serum on kinase activation.
- ^d Sample 3 lacks the fusion *trans*-activator protein and, therefore, controls for sample 4.
- ^e Sample 4 measures the effect of EGF on kinase activation.
- ^f Sample 5 controls for the extracellular stimulus as well as the fusion *trans*-activator protein.

N. Mapping signal transduction pathways using stable reporter cells.

Specific metabolic inhibitors and knockout mutant strains have played an essential role in the mapping of biochemical pathways and signal transduction pathways. The point of action of a gene product along a signal transduction pathway converging at a transcription pathway can be mapped out with stable cell lines as described here in combination with dominant negative mutants. For example, a dominant negative form of a gene that acts upstream of a particular upstream activator will not inhibit luciferase expression resulting from transient expression of an active form of the upstream activator. However, if a gene acts upstream of a dominant negative factor, the dominant negative mutant will be able to inhibit the luciferase expression. By using different genes to activate the pathway leading to pathway activation, the exact point of action of a gene product can be determined. These types of experiments have previously been performed using transient assays. The ability to make pathway-specific reporter cell lines as described herein greatly facilitates the analysis of signal transduction pathways, including determining the exact point at which an inhibitor or activator of the pathway functions.

O. Candidate Modulators According to the Invention:

A “candidate modulator” as used herein, is any compound with a potential to modulate the interaction of two proteins or the activity of a particular signal transduction pathway.

A candidate modulator is tested in a concentration range that depends upon the molecular weight of the molecule and the type of assay used. For example, for inhibition of transcription initiation, protein/DNA or protein/protein complex formation, small molecules (as defined below) may be tested in a concentration range of 1pg - 100 μ g/ml, preferably at about 100 pg - 10 ng/ml; large molecules, e.g., peptides, may be tested in the range of 10 ng - 100 μ g/ml, preferably 100 ng - 10 μ g/ml.

Inhibitors of specific signal transduction events may target a protein factor that interacts with regulatory factors so as to prevent or enhance the natural biological interaction that occurs *in vivo* which leads to transcription. As another alternative, a candidate modulator may directly affect the activity of a particular signal transduction pathway protein. Thus, a modulator of a signal transduction pathway identified as described herein will possess two properties: 1) at some concentration it will modulate the activity of a factor which is related to a specific signal transduction pathway; and 2) at the same concentration, it will not significantly affect the expression or function of unrelated factors.

Candidate modulators will include peptide and polypeptide inhibitors having an amino acid sequence based upon the components of the protein participating in the specific signal transduction pathway. For example, a mutant of a signal transduction protein factor, or a fragment of a mutant or wild-type protein, may act as a competitive inhibitor with respect to the activity of the factor.

Alternatively, a mutant or fragment of the protein may be engineered such that it instead enhances the activity of the signal transduction factor. This may be achieved through enhanced binding affinity to either its protein binding partners or through constitutive, rather than regulated activity.

A candidate modulator of the activity of the a particular signal transduction pathway, assayed as described herein, is determined to be effective if its use results in a change or difference of about 10% or greater of signal detected in the reporter assay.

The level of modulation by a candidate modulator may be quantified using any acceptable limits, for example, via the following formula. The calculations are the same when luminescent, colorimetric, fluorescent, radioactive or other detection methods are utilized.

$$\text{Percent Modulation} = \frac{(\text{Control signal} - \text{Sample signal})}{(\text{Control signal})} \times 100$$

where "Control signal" is the average of the reporter activity detected in assays that lack the candidate modulator (in other words, untreated controls), and "Sample signal" is the reporter activity detected in assays containing the candidate modulator. A similar calculation is appropriate whenever the assay yields a linear relationship between the amount of signal detected and the amount of protein or nucleic acid being represented per unit of signal or the amount of protein or nucleic acid represented by a unit of enzymatic activity.

How to Make a Reporter Cell Line According to the Invention

A reporter cell line is established by transfecting a reporter construct and a conditionally active transactivator fusion construct into a parent cell line, and selecting for clones that have integrated both the reporter and the fusion activator constructs. Clones are analyzed for background

expression levels of the reporter, inducible expression of the reporter with stimuli specific for the pathway of interest, and for the phenotypic stability of the trans-reporting system.

The primary criteria for choosing a stably transfected clone for use in the methods of the invention include stable chromosomal integration of the reporter construct, stable integration of the conditionally active fusion transactivator construct, low background of Luc or other reporter activity in the absence of activating signals, and strong, preferably dose-dependent induction of reporter activity upon treatment of cells to induce the activation of the fusion transactivation domain. The details of the establishment and evaluation of stably transfected reporter cell lines according to the invention are presented as follows.

A. Establishment of Stably Transfected Reporter Cell Lines.

Stable transfection methods are widely known in the art. Essentially, DNA is introduced to the cells by the same procedures used for transient transfection, e.g., cationic liposomes (such as LipofectAMINE™ (Life Technologies)), electroporation, calcium phosphate precipitation or DEAE dextran, followed by selection with a selection agent for that small proportion of the cells which integrate the introduced construct(s) into their chromosomes. Selection for stable transfectants is performed using a selection agent compatible with the selectable marker(s) on the plasmid(s) one desires to stably integrate.

1. Transfection and selection for stable integration.

When two different constructs are to be stably integrated, they may be either simultaneously transfected and selected for with two selection agents, or they may be sequentially transfected, selecting for clones (or pools of clones) integrating at least one copy of one construct, followed by transfection of those clones or pools of clones with the second desired construct. Because of the

relatively low occurrence frequency of stable integration events, it is often, although not always, found that the sequential approach is more effective in obtaining double stable transfectants.

A sample transfection scheme is outlined below.

Day 1: Plate cells in 100 mm tissue culture dishes (5×10^5 cells per dish).

Day 2: Transfect cells using the Mammalian Transfection Kit (Stratagene), with 1-10 ug of plasmid DNA per transfection as follows:

a) prepare a DNA-lipid complex according to the instructions supplied by the manufacturer of the polycationic liposome transfection kit;

b) dilute the DNA-lipid complex to a final volume of 3 ml with serum-free medium;

c) rinse the cells with serum-free medium;

d) mix gently and overlay 1 ml of the diluted DNA-lipid complex onto the cells;

e) incubate the cells with the DNA-lipid complex for 5-7 hours (this is an estimate; the time of exposure of cells to lipid-DNA complex will vary with cell type and the exact amount and ratio of DNA and lipid; one of skill in the art may determine the optimal amounts of DNA and lipid, the optimal duration of cell exposure, as well as whether medium should be supplemented with small amounts of serum during the exposure of cells to the DNA-lipid complex, through a series of preliminary experiments wherein each variable is systematically varied while all others are held constant; determinations of this kind are routine in the field);

f) rinse off the DNA-lipid complex and add complete non-selective medium.

Day 3: Wash cells twice with PBS. Add fresh non-selective medium.

Day 4: Split cells 1: 10 - 1:80 into 100 mm petri dishes.

Day 5: Change to selective medium containing selective agent (e.g., 0.5 mg/ml hygromycin or 0.2

mg/ml G418; amounts may be varied depending on the sensitivity of a given cell line to the chosen selective agent(s) as determined by one of skill in the art).

Day 15: (14 - 20 days are required for single, well-isolated colonies to be visible) Single colonies are lifted from the plates using small pieces of sterile filter paper saturated with Trypsin/EDTA. Single colonies are transferred to 24-well plates. As these wells become confluent, the clones are expanded to 12-well, and then 6-well plates, and finally to T-25 tissue culture flasks. At this point the selective pressure may be reduced by 50% (0.25 mg/ml G418 and/or 0.1 mg/ml hygromycin). The clones are then tested by transient transfection and, if positive, frozen for storage.

Stable integration is determined by PCR amplification of a band of the expected size using primers specific for portions of the transfected constructs as described herein.

Resulting stable colonies are picked and screened to determine background expression of the reporter, and to evaluate the response of the reporter to expression of transactivators or fusion transactivators. Clones that demonstrate the strongest responses may then be tested more extensively.

2. Evaluating the function of stably integrated constructs.

Clones with integrated reporter and fusion transactivator constructs must be functionally evaluated with regard to the pathway-specific reporting system. Specifically, cells are evaluated for reporter background activity and for the response of the reporter to treatments that induce the pathway to which the reporter/fusion transactivator system is designed to respond.

Clones of cells bearing stably integrated reporter and fusion transactivator constructs are evaluated for background activity of the reporter by assaying the reporter in cells without any treatment to induce the activation of the fusion transactivation domain.

Doubly transfected clones exhibiting low background are then tested for the response of the reporter to activating signals. Activating signals may be any of those discussed in the section "Activating Signals" above, including, but not limited to growth factors, cytokines, chemical agents (such as cycloheximide or PMA), treatments such as UV or other irradiation, treatments that induce thermal, oxidative, or hypoxic stress, and expression of upstream activators. It is expected that the reporter in pathway-specific reporter cell lines will respond to only those activators that normally activate the pathway the cells were designed to monitor. Assays with activating signals may be performed with varying doses or amounts of the activating signal to monitor the dose-dependence of the reporter response.

B. Assessing the Stability of Stably Transfected Constructs.

The phenotypic stability of the integrated construct(s) in cells of the invention may be assessed by treating cells from a given clone as follows: one group of cells from a clone may be continuously maintained in culture (under selective pressure, such as 50% of the level of selective agent(s) used in the original selection for the clones) for two months; another group may be subjected to the cycle of freezing, storage at -80°C, thawing and reculturing at least two times during a two month period; and a third group may be frozen and thawed as was the second group, except with storage under liquid nitrogen. Cells treated in this manner are then tested for reporter expression in response to transient transfection with upstream activators and/or in response to extracellular stimuli such as activating chemical or growth factor treatment. Cells that continue to exhibit a reporter response to such activating treatments after the various storage and culture regimens are considered to have a stable phenotype according to the invention.

C. Selection of Stably Transfected Reporter Cell Lines Using HeLa Cells as the Parental Cell Line.

The *trans*-reporting systems described herein have been tested by transient assays in HeLa cells. As such, the HeLa cell line was used as a parent cell line to establish stable reporter cell lines.

HeLa cells cultured as described above were stably transfected as follows (this scheme is similar for both single and double transfectants, with the difference being that double transfectants are selected with two selective agents, instead of one as used for single transfectants):

Day 1: Plate cells in 100 mm tissue culture dishes (5×10^5 cells per dish).

Day 2: Transfect cells using the Mammalian Transfection Kit (Stratagene), with 1-10 μ g of plasmid DNA per transfection.

Day 3: Wash cells twice with PBS. Add fresh non-selective medium (or, for cells already bearing a single stable construct and being transfected with a second construct, add fresh medium supplemented with only the selective agent for the first construct).

Day 4: Split cells 1:10 - 1:80 into 100 mm petri dishes.

Day 5: Change to selective medium containing 0.2 mg/ml hygromycin.

Day 15: (14 - 20 days are required for single, well-isolated colonies to be visible) Single colonies were lifted from the plates using small pieces of sterile filter paper saturated with Trypsin/EDTA. Single colonies were transferred to 24-well plates. As these wells became confluent, the clones were expanded to 12-well, and then 6-well plates, and finally to T-25 tissue culture flasks. At this point the selective pressure was reduced by 50% (0.25 mg/ml G418 and/or 0.1 mg/ml hygromycin). The clones were then tested by transient transfection and, if positive, frozen for storage.

After transfection of HeLa cells with pFR-Luc-Hyg, a total of 11 hygromycin-resistant clones

were selected and tested for reporter background. One clone, #1C3, showed very low background reporter expression (data not shown). Clone #1C3 was then tested for its response to activation of reporter expression.

Transient transfection assays to identify clones responsive to appropriate upstream stimuli include a complete transactivator construct (i.e., a construct encoding a transactivation domain and a DNA binding domain recognizing the DNA binding recognition element on the reporter. Alternatively, an appropriate fusion transactivator may be transiently transfected along with a plasmid encoding an upstream activating factor. Clone #1C3, for example, was highly responsive to transient transfection with plasmid pFC-MEKK, encoding the upstream activator MEKK and the fusion transactivator encoded by pFA2-Jun (see Figure 6). Therefore, clone # 1C3 was selected for later use and designated RCLH-Luc (for **r**eporter **c**ell line derived from **H**eLa containing **l**uciferase). All of the stable, doubly transfected cell lines containing both pFR-Luc and fusion transactivator vectors (see below) were established by transfecting the latter plasmid into the RCLH-Luc reporter cell line.

D. Establishment of Pathway-Specific Reporter Cell Lines.

Several approaches have been tried to simultaneously transfect both the reporter vector pFR-Luc-Hyg and the conditionally active, pathway-specific fusion transactivator vectors into HeLa cells. Only the sequential approach of transfecting cells already stably transfected with pFR-Luc-Hyg (i.e., RCLH-Luc cells) with the fusion transactivator plasmid vector and selecting with G418 (0.5 mg/ml) turned out to be successful (see Figure 3 for a schematic of the approach taken in transfecting HeLa cells with the pFR-Luc reporter and a pathway-specific fusion transactivator).

G418-resistant clones are screened for integration of the fusion transactivator construct by

PCR, with one primer specific to the GAL4 DNA binding domain and the other to the fusion transactivation domain. Detection of an amplified fragment of the expected size confirms the chromosomal integration of the fusion transactivator construct.

Kits According to the Invention

In one embodiment of the invention, a single or double stably transfected reporter cell line may be assembled into a kit, such a kit allowing one to assay protein:protein interactions, the activity of specific signal transduction pathways, the activity of specific transactivating proteins or transactivation domains thereof and/or to screen candidate modulators of the activity of such pathways or transactivating proteins or domains thereof. A kit of this type will comprise a reporter cell line of the invention, packaging materials and instructions for the use thereof. A kit may additionally comprise activating signal compounds or nucleic acid constructs (such as those encoding activator- or DNA binding domain-fusion proteins or upstream activator proteins specific to a given signal transduction pathway) as described herein above.

ATCC DEPOSIT

The reporter cell lines RCLH-Elk1, -c-Jun, -CHOP, and -Creb disclosed herein has been deposited with American Type Culture Collection under deposit numbers [insert numbers]. ATCC deposit numbers [insert numbers] were deposited under the terms of the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia, 20110-2209 on [insert date], and the deposit will be maintained for 30 years from the date of deposit and during the pendency of a patent that issues thereon.

The cell lines and methods of the invention are further described in the following non-limiting examples, and in the claims.

EXAMPLES

Example 1. Elk1 Pathway-Specific Cell Lines

A. Establishment of Elk1 Pathway-Specific Cell Lines.

Several approaches were tried initially to simultaneously transfect both the reporter vector pFR-Luc-Hyg and the conditionally active, pathway-specific fusion transactivator vector pFA2-Elk into HeLa cells. Only the sequential approach of transfecting cells already stably transfected with pFR-Luc-Hyg (i.e., RCLH-Luc cells) with the pFA2-Elk1 plasmid vector and selecting with G418 (0.5 mg/ml) turned out to be successful (see Figure 3 for a schematic of the approach taken in transfecting HeLa cells with the pFR-Luc reporter and the Elk1 pathway fusion transactivator).

G418-resistant clones were screened for integration of the pFA2-Elk1 construct by PCR, with one primer specific to the GAL4 DNA binding domain and the other to the Elk1 transactivation domain. Detection of an amplified fragment of the expected size confirms the chromosomal integration of the pFA2-Elk fusion transactivator construct.

The resulting clones containing integrated pFA-2 Elk1 were initially screened by their response to the transiently transfected positive control upstream activator vector pFC-MEK1. A total of nine G418 resistant clones were screened with assays similar to those shown in Figure 6, and clone 1-10 was selected for further testing. Criteria for choosing clone 1-10 over the other cell lines include stable integration of pFR-Luc and pFA2-Elk1, low background of Luc expression in the absence of signals which activate the activator fusion protein, and strong, dose-dependent induction of reporter activity upon treatment of cells to induce the activity of the activator fusion protein.

09637650 "081100

If the chromosomally integrated pFA2-Elk1 functions properly in RCLH-Elk1 cells, stimulation of endogenous MAPK should result in the activation GAL4-Elk1 and, consequently, luciferase expression from chromosomal copies of pFR-Luc. Luciferase activity in RCLH-Elk1 cells was indeed enhanced as much as 100-fold by the overexpression of MEK 1, a known activator of MAPK (Fig 7). This increase was dose-dependent, with the fold activation of luciferase expression increasing with increasing doses of MEK1 expression plasmid (pFC-MEK1, available from Stratagene), peaking at around 10 ng/well and declining thereafter with increasing amounts of MEK1 expression plasmid (Fig 7).

Overexpression of a protein (e.g. MEK1) in the cell usually gives rise to more dramatic and sometimes non-physiological results. Therefore, RCLH-Elk1 cells were subjected to further testing with extracellular stimuli known to activate MAPK. Epidermal growth factor (EGF) and phorbol 12-myristate 13-acetate (PMA), also known as 12-O-tetradecanoyl-13acetate (TPA), are well documented MAPK activators. Luciferase expression in RCLH-Elk1 cells was 5 times as high as the untreated control when the cells were treated with 100 ng/ml of EGF and was almost 50 fold higher when the cells were challenged with PMA (60 ng/ml) (Fig 8). The effects of EGF and PMA seem to be additive (Fig. 11), supporting the notion that they activate MAPK via different pathways. The rather moderate effect of EGF in this particular experiment was most likely caused by a suboptimal dose of EGF, as it had been stored for an extended period of time and was subjected to several freeze/thaw cycles.

The phenotypic stability of the transreporting system in the RCLH-Elk1 cells was evaluated as follows. RCLH-Elk1 stable cell lines were frozen and thawed two times over a period of two months. Following each freeze/thaw cycle, cells were tested for their response to

the transiently transfected upstream activator plasmid pFC-MEK1. These tests indicated that the pathway-specific reporting system in the RCLH-Elk1 cells was retained through cycles of freezing, thawing and re-culturing. RCLH-Elk1 cells from the first and the second freeze/thaw cycles have also been propagated in continuous culture for as long as five months. Transfection of upstream activator continued to induce reporter expression even after this length of time in continuous culture. Therefore, the phenotype with respect to the reporter system is stable in these cell lines.

It should be noted that the absolute level of reporter induction will vary depending upon the number of copies of integrated reporter and fusion transactivator plasmids. Transient transfection generally introduces more copies of the respective plasmids than are retained in stable cell lines. Therefore, the absolute reporter activity level obtained from stable reporter cell lines is generally lower than in similar transient assays. The fold activation, however, generally remains similar whether using transient or stable transfection, since transient assays have higher reporter background than do assays in stable cells. For example, when pFA2-Elk1 is transiently transfected into HeLa cells together with pFR-Luc and pFC-MEK1, the maximum luciferase activity observed was 3,610,540 RLU with a background control value of 115,000 RLU. This represents a 31-fold activation. In the case of RCLH-Elk1 cells, the peak luciferase activity was 236,276 RLU with a background of 4,692 RLU - a 50-fold activation.

Taken together, these results demonstrated that the chromosomally integrated copies of both pFR-Luc and pFA2-Elk1 reconstitute the pathway-specific reporter assay system in the RCLH-Elk1 stable cells, and that the system is phenotypically stable. Signal transduction pathways converging at the MAPK or Elk1 proteins can be studied much more extensively,

conveniently and consistently with RCLH-Elk1 cells than with the transient system. As the name mitogen activated protein kinase suggests, the MAPK pathway plays a pivotal role in normal, as well as abnormal, cell growth and differentiation. Many components along the pathway are known oncogenes and potential targets of various drug-screening efforts. Therefore, RCLH-Elk1 cells will greatly enhance the ability to set up high throughput assays needed for drug (inhibitors) screening efforts and panel assays for possible carcinogenic activities (activators) of various commercial compounds.

B. Analysis of the function of a gene product using RCLH-Elk stable reporter cells.

To study if MEK1 is involved in the MAPK activation pathway, a vector expressing constitutively active MEK1 (pCA-MEK1) was transiently transfected into RCLH-Elk1 cells (see Figure 9). As compared with the vector alone, the expression of CA-MEK1 greatly increased luciferase expression from the integrated luciferase construct. Therefore, it can be concluded from this experiment that MEK1 is involved in the signal transduction pathway(s) converging at Elk1. It can also be concluded that the protein kinase activity of MEK1 is also required for its function in this pathway, since the expression of a dominant negative MEK1 mutant deficient in kinase activity (DN-MEK1) did not activate luciferase expression significantly. Indeed, DN-MEK1 acted as a dominant negative inhibitor of MAPK pathway, inhibiting the luciferase expression induced by the constitutive activator CA-MEK1.

As an extension of the method of investigating the effect of a gene product on a signal transduction pathway, one may also screen individual clones from an expression library or mutants of known regulatory factors for their effect on a signal transduction pathway. Methods of generating and using libraries as well as methods of mutagenesis are known in the art. See for

example, Ausubel et al. (1988, supra), and Sambrook et al. (1989, supra).

C. Screening For Activators of the Elk1 Pathway with RCLH-Elk1 Cells

The following study demonstrates the use of RCLH-Elk1 stable reporter cells to screen for activators of the MAPK pathway converging on Elk1. Because RCLH-Elk1 cells constitutively express the GAL4-Elk1 fusion, there is no transfection of any foreign DNA required to screen for extracellular stimuli or chemical compounds that can activate components along the MAPK signal transduction pathway. One need only grow the RCLH-Elk1 cells, treat them with the stimulus in question and analyze the expression of luciferase. Figure 10 shows the results of screening RCLH-Elk1 cells with DMSO, anisomycin, forskolin plus IBMX, EGF, PMA, or PMA plus EGF. From this experiment, it can be concluded that both PMA and EGF are activators of MAPK pathways leading to Elk1 activation while DMSO, anisomycin and forskolin plus IBMX are not. A separate experiment indicated that TGF- α also activates MAPK pathways.

D. Mapping signal transduction pathways using RCLH-Elk stable reporter cells.

Specific metabolic inhibitors and knockout mutant strains have played an essential role in the mapping of biochemical pathways and signal transduction pathways. The point of action of a gene product along a signal transduction pathway converging at a transcription pathway can be mapped out with stable cell lines described here in combination with dominant negative mutants. In the above Example, GAL4-Elk1 fusion protein in the RCLH-Elk1 cells was activated by CA-MEK1. A dominant negative form of a gene that acts upstream of MEK1 (e.g. c-Raf) will not inhibit luciferase expression resulting from the activation of the MAPK pathway in the cell. However, if a gene acts downstream of MEK1 (e.g. ERK1), the dominant negative mutant of this gene will be able to inhibit the luciferase expression. By using different genes to activate the

pathway leading to Elk1 activation, the exact point of action of a gene product can be determined. These types of experiments have previously been performed using transient assays. The ability to make pathway-specific reporter cell lines as described herein greatly facilitates the analysis of signal transduction pathways, including determining the exact point at which an inhibitor or activator of the pathway functions.

clones were screened with assays similar to those shown in Figure 11, and clone #1-8 was selected for further testing. Criteria for choosing clone #1-8 over the other cell lines include stable integration of pFR-Luc and pFA2-CREB, low background of Luc expression in the absence of signals which activate the activator fusion protein, and strong, dose-dependent induction of reporter activity upon treatment of cells to induce the activity of the activator fusion protein (see below).

Expression of the catalytic subunit of murine PKA in RCLH-CREB cells resulted in as much as a 75-fold increase in luciferase activity. The activation of luciferase expression increased with increasing doses of the pFC-PKA activator expression plasmid, peaking at around 10 ng/well, and declined thereafter with increasing amounts of activator plasmid (Figure 12). Therefore, the luciferase activity in RCLH-CREB cells can reflect the level of PKA activity inside the cells.

The effects of various cAMP elevating agents on luciferase reporter expression were evaluated in transient transfection assays using the pFR-Luc and pFA2-CREB plasmids (data not shown). Compounds tested include dibutyryl cAMP, cholera toxin (CT toxin), adenylyl cyclase toxin (AC toxin), 3-isobutyl-1-methylxanthine (IBMX) and forskolin. Dibutyryl cAMP is membrane permeable and directly elevates cellular cAMP level. AC toxin itself has cyclase activity while CT toxin activates cellular adenylyl cyclase activity by inhibiting GTPase activity. Forskolin directly activates adenylyl cyclase and IBMX inhibits cAMP phosphodiesterase activity. Although these compounds elevate cellular cAMP level via distinct mechanisms, they all stimulated luciferase expression from the pFA-CREB transreporting systems, with responses ranging from 3-13 fold in transient assays, with AC toxin having the strongest effect (13 fold at

10 ug/ml; data not shown).

Overexpression of a protein (e.g., PKA) in the cell often gives rise to dramatic, but sometimes non-physiological results. Therefore, the effect of extracellular stimuli affecting the cAMP/CREB pathway was examined using RCLH-CREB cells. As shown in Figure 13, treatment of RCLH-CREB cells with 50 ug/ml of AC toxin, among the compounds mentioned above, gave rise to strong effect on luciferase expression (approximately 7 fold) consistent with the result from transient assays. Phobol ester (PMA) stimulated luciferase expression from RCLH-CREB cells dramatically (approximately 70 fold) in the presence of 100 uM forskolin. This effect of PMA has been observed in cell lines containing a construct similar to pCRE-Luc (George et al., 1997, J. Biomol. Screening 2: 235-240). Because PMA has no effect on cellular cAMP level, the effect of PMA on CREB or CRE is believed to be a result of crosstalk between cAMP and other signaling pathways (George et al., 1997, supra). Dibutyryl cAMP, forskolin and IBMX all induced luciferase activity by themselves, consistent with their specifically activating the cAMP-mediated CREB pathway, however, they increased luciferase activity less than 3 fold that of the controls under the conditions used (not shown).

The phenotypic stability of the transreporting system in the RCLH-CREB cells was evaluated as follows. RCLH-CREB stable cell lines were frozen and thawed two times over a period of two months. Following each freeze/thaw cycle, cells were tested for their response to the transiently transfected upstream activator plasmid pFC-PKA. These tests indicated that the pathway-specific reporting system in the RCLH-CREB cells was retained through cycles of freezing, thawing and re-culturing. RCLH-CREB cells from the first and the second freeze/thaw cycles have also been propagated in continuous culture for as long as five months. Transfection

of upstream activator continued to induce reporter expression even after this length of time in continuous culture. Therefore, the phenotype with respect to the reporter system is stable in these cell lines.

Taken together, these results demonstrated that the chromosomally integrated copies of both pFR-Luc and pFA2-CREB reconstitute the pathway-specific reporter assay system in the RCLH-CREB stable cells, and that the system is phenotypically stable. Signal transduction pathways converging at the CREB protein can be studied much more extensively, conveniently and consistently with RCLH-CREB cells than with the transient system. Components along the CREB pathway are potential targets of drug-screening efforts. Therefore, RCLH-CREB cells will greatly enhance the ability to set up high throughput assays needed for drug (inhibitors) screening efforts and panel assays for possible carcinogenic activities (activators) of various commercial compounds.

Example 3. CHOP Reporter Cell Lines

A. Establishment of CHOP Reporter Cell Lines

Several approaches were tried initially to simultaneously transfect both the reporter vector pFR-Luc-Hyg and the conditionally active, pathway-specific fusion transactivator vector pFA2-CHOP into HeLa cells. Only the sequential approach of transfecting cells already stably transfected with pFR-Luc-Hyg (i.e., RCLH-Luc cells) with the pFA2-CHOP plasmid vector and selecting with G418 (0.5 mg/ml) turned out to be successful (see Figure 3 for a schematic of the approach taken in transfecting HeLa cells with the pFR-Luc reporter and the CHOP pathway fusion transactivator).

G418-resistant clones were screened for integration of the pFA2-CHOP construct by

PCR, with one primer specific to the GAL4 DNA binding domain and the other to the CHOP transactivation domain. Detection of an amplified fragment of the expected size confirms the chromosomal integration of the pFA2-CHOP fusion transactivator construct.

The resulting clones containing integrated pFA-2 CHOP were initially screened by their response to the transiently transfected positive control upstream activator vector pFC-MEK3. A total of thirty-one G418 resistant clones were screened with assays similar to those shown in Figure 14, and clone #1-4 was selected for further testing. Criteria for choosing clone #1-4 over the other cell lines include stable integration of pFR-Luc and pFA2-CHOP, low background of Luc expression in the absence of signals which activate the activator fusion protein, and strong, dose-dependent induction of reporter activity upon treatment of cells to induce the activity of the activator fusion protein.

The dose-dependence of RCLH-CHOP clone #1-4 reporter activation was evaluated by transient transfection with varying amounts of pFC-MEK3 as shown in Figure 15. RCLH-CHOP clone #1-4 exhibited a strong response with 25 ng/well of pFC-MEK3 expression plasmid, followed by a decline in activation at 50 and 100 ng/well. followed by

Overexpression of a protein (e.g., MEK3) in the cell often gives rise to dramatic, but sometimes non-physiological results. The effect of extracellular stimuli affecting the CHOP pathway may be examined using RCLH-CHOP cell lines. For example, RCLH-CHOP cells may be treated with UV light or TNF- α , which are known to activate the p38 MAPK/CHOP pathway, followed by luciferase assays. The response of the chromosomally integrated reporter to extracellular stimuli which activate the p38 MAPK/CHOP pathway indicates that the chromosomally integrated copies of the reporter and fusion transactivator constructs reconstitute

the CHOP-specific reporter assay system in the RCLH-CHOP stable cells.

The phenotypic stability of the transreporting system in the RCLH-CHOP cells was evaluated as follows. RCLH-CHOP stable cell lines were either continuously cultured, stored at -80°C, or stored under liquid nitrogen for a period of over three months. Cells were then assayed for luciferase activity in response to pFC-MEK3 transfection. As shown in Figure 16, cells retained responsiveness to transfected upstream activator. Storage in liquid nitrogen preserved the responsive phenotype, including the dose-responsiveness, to a greater extent than did the other storage or culture conditions. Nonetheless, the RCLH-CHOP reporter cell phenotype is stable.

Taken together, these results demonstrated that the chromosomally integrated copies of both pFR-Luc and pFA2-CHOP reconstitute the pathway-specific reporter assay system in the RCLH-CHOP stable cells, and that the system is phenotypically stable. Signal transduction pathways converging at the CHOP protein can be studied much more extensively, conveniently and consistently with RCLH-CHOP cells than with the transient system. As the name mitogen-activated protein kinase suggests, the MAPK pathway plays a pivotal role in normal, as well as abnormal, cell growth and differentiation. Many components along the pathway are known oncogenes and potential targets of various drug-screening efforts. Therefore, RCLH-CHOP cells will greatly enhance the ability to set up high throughput assays needed for drug (inhibitors) screening efforts and panel assays for possible carcinogenic activities (activators) of various commercial compounds.

Example 4. c-Jun Reporter Cell Lines

A. Establishment of RCLH-c-Jun cell lines.

Several approaches were tried initially to simultaneously transfect both the reporter vector pFR-Luc-Hyg and the conditionally active, pathway-specific fusion transactivator vector pFA2-Jun into HeLa cells. Only the sequential approach of transfecting cells already stably transfected with pFR-Luc-Hyg (I.e., RCLH-Luc cells) with the pFA2-Jun plasmid vector and selecting with G418 (0.5 mg/ml) turned out to be successful (see Figure 3 for a schematic of the approach taken in transfecting HeLa cells with the pFR-Luc reporter and the c-Jun pathway fusion transactivator).

G418-resistant clones were screened for integration of the pFA2-Jun construct by PCR, with one primer specific to the GAL4 DNA binding domain and the other to the c-Jun transactivation domain. Detection of an amplified fragment of the expected size confirmed the chromosomal integration of the pFA2-Jun fusion transactivator construct.

The resulting clones containing integrated pFA2-Jun were initially screened for their response to the transiently transfected positive control upstream activator vector pFC-MEKK. A total of 9 G418 resistant clones were screened, and clone #1-4 was selected for further testing. The dose-dependence of the reporter response in clone #1-4 was investigated by transient transfection with varying doses of pFC-MEKK as shown in Figure 17. Criteria for choosing clone #1-4 over the other cell lines include stable integration of pFR-Luc and pFA2-Jun, low background of Luc expression in the absence of signals which activate the activator fusion protein, and strong, dose-dependent induction of reporter activity upon treatment of cells to induce the activity of the activator fusion protein.

Overexpression of a protein (e.g. MEKK) in the cell usually gives rise to more dramatic and sometimes non-physiological results. In order to test the response of the integrated reporter

system to extracellular stimuli, the RCLH-cJun cells may be treated with extracellular stimuli known either to stimulate (e.g., UV, TNF α) or not to stimulate (e.g., PDGF or PMA treatment) the JNK pathway, followed by reporter assays.

The phenotypic stability of the transreporting system in the RCLH-cJun cells was evaluated as follows. RCLH-cJun stable cell lines were either continuously cultured for over three months (splitting two times a week), stored frozen at -80°C, or stored frozen under liquid nitrogen, were assayed for their responses to transient transfection with pFC-MEKK. As shown in Figure 18, RCLH-cJun cells continued to respond to stimulation with MEKK, regardless of the storage of culture regimen. Therefore, the phenotype with respect to the reporter system is stable in these cell lines.

It should be noted that the absolute level of reporter induction will vary depending upon the number of copies of integrated reporter and fusion transactivator plasmids. Transient transfection generally introduces more copies of the respective plasmids than are retained in stable cell lines. Therefore, the absolute reporter activity level obtained from stable reporter cell lines is generally lower than in similar transient assays. The fold activation, however, generally remains similar whether using transient or stable transfection, since transient assays have higher reporter background than do assays in stable cells.

Signal transduction pathways converging at the JNK or c-Jun proteins can be studied much more extensively, conveniently and consistently with RCLH-cJun cells than with a transient transfection reporter system. c-Jun was originally identified as the cellular homolog of the v-Jun oncogene. Deregulation of pathways leading to the activation of c-Jun can play a role in tumorigenesis. RCLH-cJun cells of the invention permit the establishment of high throughput

assays for drug screening efforts (i.e., to identify inhibitors) and panel assays for possible carcinogenic activities of various commercial compounds (i.e., to identify activators).

Example 5: Assay of protein:protein interaction in mammalian cells.

In addition to being used as a starter cell line for the double stable cells, singly transfected stable reporter cell lines, such as RCLH-Luc, can serve as the reporter cell line for many other applications using the GAL4 DNA or other binding domain. One of these applications is to test protein-protein interactions with mammalian two-hybrid vectors. In these systems, one protein is fused to the selected DBD and the other protein is fused to the activation domain of a transcription factor. If these two proteins interact with each other physically, the activation domain will be recruited through this protein-protein interaction to the vicinity of the operably linked DBD recognition sequence elements and activate transcription of the integrated reporter. To test this method, the following experiment was performed.

The SV40 large T antigen is known to interact with p53. Therefore, a vector encoding an SV40 large T antigen-GAL4 DBD fusion protein and a vector encoding a p53-GAL4 AD fusion protein were transfected either alone, or in combination into RCLH-Luc cells. As shown in Figure 19, luciferase was expressed from the chromosomally incorporated pFR-Luc plasmid when expression vectors for GAL4 DBD/large T antigen and of GAL4 AD/p53 fusion proteins were introduced into the cell together (bar labeled p53 + SV40). Only background luciferase expression was observed when either one of the vectors was introduced separately (bars labeled p53 + AD and BD + SV40).

OTHER EMBODIMENTS

Parameter	Value	Unit
Initial temperature	25.0	°C
Final temperature	25.0	°C
Initial pressure	1.013	bar
Final pressure	1.013	bar
Initial volume	0.001	m³
Final volume	0.001	m³
Initial mass	0.001	kg
Final mass	0.001	kg
Initial density	1000	kg/m³
Final density	1000	kg/m³
Initial viscosity	0.001	Pa·s
Final viscosity	0.001	Pa·s
Initial thermal conductivity	0.6	W/m·K
Final thermal conductivity	0.6	W/m·K
Initial specific heat capacity	4182	J/kg·K
Final specific heat capacity	4182	J/kg·K
Initial enthalpy	0	J
Final enthalpy	0	J
Initial entropy	0	J/K
Final entropy	0	J/K
Initial internal energy	0	J
Final internal energy	0	J
Initial kinetic energy	0	J
Final kinetic energy	0	J
Initial potential energy	0	J
Final potential energy	0	J
Initial total energy	0	J
Final total energy	0	J
Initial momentum	0	kg·m/s
Final momentum	0	kg·m/s
Initial torque	0	N·m
Final torque	0	N·m
Initial power	0	W
Final power	0	W
Initial force	0	N
Final force	0	N
Initial stress	0	Pa
Final stress	0	Pa
Initial strain	0	-
Final strain	0	-
Initial displacement	0	m
Final displacement	0	m
Initial velocity	0	m/s
Final velocity	0	m/s
Initial acceleration	0	m/s²
Final acceleration	0	m/s²
Initial angular velocity	0	rad/s
Final angular velocity	0	rad/s
Initial angular acceleration	0	rad/s²
Final angular acceleration	0	rad/s²
Initial rotation	0	rad
Final rotation	0	rad
Initial frequency	0	Hz
Final frequency	0	Hz
Initial period	0	s
Final period	0	s
Initial wavelength	0	m
Final wavelength	0	m
Initial wave number	0	1/m
Final wave number	0	1/m
Initial phase	0	rad
Final phase	0	rad
Initial amplitude	0	m
Final amplitude	0	m
Initial frequency spectrum	0	1/Hz
Final frequency spectrum	0	1/Hz
Initial power spectrum	0	W/Hz
Final power spectrum	0	W/Hz
Initial force spectrum	0	N/Hz
Final force spectrum	0	N/Hz
Initial stress spectrum	0	Pa/Hz
Final stress spectrum	0	Pa/Hz
Initial strain spectrum	0	-/Hz
Final strain spectrum	0	-/Hz
Initial displacement spectrum	0	m/Hz
Final displacement spectrum	0	m/Hz
Initial velocity spectrum	0	m/s/Hz
Final velocity spectrum	0	m/s/Hz
Initial acceleration spectrum	0	m/s²/Hz
Final acceleration spectrum	0	m/s²/Hz
Initial angular velocity spectrum	0	rad/s/Hz
Final angular velocity spectrum	0	rad/s/Hz
Initial angular acceleration spectrum	0	rad/s²/Hz
Final angular acceleration spectrum	0	rad/s²/Hz
Initial rotation spectrum	0	rad/Hz
Final rotation spectrum	0	rad/Hz
Initial frequency spectrum	0	1/Hz
Final frequency spectrum	0	1/Hz
Initial power spectrum	0	W/Hz
Final power spectrum	0	W/Hz
Initial force spectrum	0	N/Hz
Final force spectrum	0	N/Hz
Initial stress spectrum	0	Pa/Hz
Final stress spectrum	0	Pa/Hz
Initial strain spectrum	0	-/Hz
Final strain spectrum	0	-/Hz
Initial displacement spectrum	0	m/Hz
Final displacement spectrum	0	m/Hz
Initial velocity spectrum	0	m/s/Hz
Final velocity spectrum	0	m/s/Hz
Initial acceleration spectrum	0	m/s²/Hz
Final acceleration spectrum	0	m/s²/Hz
Initial angular velocity spectrum	0	rad/s/Hz
Final angular velocity spectrum	0	rad/s/Hz
Initial angular acceleration spectrum	0	rad/s²/Hz
Final angular acceleration spectrum	0	rad/s²/Hz
Initial rotation spectrum	0	rad/Hz
Final rotation spectrum	0	rad/Hz

CLAIMS

1. A cell line comprising a stably integrated recombinant nucleic acid construct comprising:
 - a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and
 - a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, the fusion protein comprising a sequence-specific DNA binding domain, wherein the DNA binding domain specifically binds said recognition sequence, and a conditionally active transactivation domain, wherein activation of said conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, and wherein binding of said fusion protein to said recognition sequence results in transactivation of said reporter gene when said transactivation domain fused to said DNA binding domain is activated.
2. The cell line of claim 1, wherein said reporter gene is selected from the group consisting of luciferase, β -galactosidase, chloramphenicol acetyltransferase, secreted alkaline phosphatase and green fluorescent protein.
3. The cell line of claim 2, wherein said reporter gene is luciferase.
4. The cell line of claim 1, wherein said recognition sequence for a sequence-specific DNA-binding domain is that sequence recognized by one of the group consisting of GAL4

and LexA.

5. The cell line of claim 1 wherein said fusion protein is constitutively expressed.

6. The cell line of claim 1 wherein said fusion protein is constitutively expressed in a specific cell type.

7. The cell line of claim 1, wherein the cell line is of mammalian origin.

8. The cell line of claim 1, wherein said parent cell line is human.

9. The cell line of claim 1, wherein said parent cell line is HeLa.

10. A method of assaying for the activity of a signal transduction pathway in a mammalian cell, said method comprising the steps of:

detecting in a signal transduction pathway-specific reporter cell line expression of a reporter gene, wherein said reporter cell line comprises:

a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and

a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, said fusion protein comprising a sequence-specific DNA binding domain, wherein said DNA binding domain specifically binds said recognition sequence,

and a conditionally active transactivation domain, wherein activation of said conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, wherein binding of said fusion protein to said recognition sequence results in transactivation of said reporter gene when said transactivation domain fused to said DNA binding domain is activated, wherein expression of said reporter gene is indicative of activity of said signal transduction pathway.

11. A method of screening for a modulator of the activation of a signal transduction pathway in a mammalian cell, said method comprising the steps of:

(a) contacting a stable reporter cell line with a candidate modulator under conditions sufficient to permit activation of said signal transduction pathway, the reporter cell line comprising:

a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and

a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, said fusion protein comprising a sequence-specific DNA binding domain, wherein said DNA binding domain specifically binds said recognition sequence, and a conditionally active transactivation domain, wherein activation of said conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, wherein binding of said fusion protein to said recognition sequence results in transactivation of said reporter gene when said transactivation domain fused to said DNA binding domain is activated; and

(b) detecting expression of said reporter gene, wherein a difference in expression of said reporter gene in the presence of said candidate modulator and in the absence of said candidate modulator is indicative of modulatory activity of said candidate modulator on said pathway.

12. The method of claim 11, further comprising, during said contacting step, providing an activator signal, said signal activating said conditionally active transactivation domain.

13. The method of claim 12, wherein said providing comprises adding an activator compound to the culture medium of said reporter cell line.

14. The method of either one of claims 10 or 11 wherein said DNA binding domain is selected from the group consisting of the DNA binding domains of GAL4 and LexA.

15. The method of either one of claims 10 or 11 wherein said mammalian cell is human.

16. The method of claim 15 wherein said mammalian cell is a HeLa cell.

17. A method of assaying for the activation of a conditionally active transactivation domain in a mammalian cell, said method comprising the steps of:

00180-0592960

a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, said fusion protein comprising a sequence-specific DNA binding domain, wherein said DNA binding domain specifically binds said recognition sequence, and a conditionally active transactivation domain, wherein activation of said conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, wherein binding of said fusion protein to said recognition sequence results in transactivation of said reporter gene when said transactivation domain fused to said DNA binding domain is activated; and

(b) detecting the expression of said reporter gene, wherein a difference in expression of said reporter gene in the presence of said candidate modulator and in the absence of said candidate modulator is indicative of modulatory activity of said candidate modulator on said conditionally active transactivating protein.

19. The method of claim 18, further comprising, during said contacting step, providing an activator signal, said signal activating said conditionally active transactivation domain.

20. The method of claim 19, wherein said providing comprises adding an activator compound to the culture medium of said reporter cell line.

21. The method of either one of claims 17 or 18 wherein said DNA binding domain is selected from the group consisting of the DNA binding domains of GAL4 and LexA.

22. The method of either one of claims 17 or 18 wherein said mammalian cell is human.

23. The method of claim 22 wherein said mammalian cell is a HeLa cell.

24. A kit comprising the cell line of claim 1 and packaging therefor.

25. A kit for performing the method of any one of claims 10, 11, 17 or 18, said kit comprising a cell line comprising a stably integrated recombinant nucleic acid construct comprising:

a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein; and

a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, said fusion protein comprising a sequence-specific DNA binding domain, wherein said DNA binding domain specifically binds said recognition sequence, and a conditionally active transactivation domain, wherein activation of said conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, and wherein binding of said fusion protein to said recognition sequence results in transactivation of said reporter gene when said transactivation domain fused to said DNA binding domain is activated.

26. The kit of claim 25, said kit further comprising a nucleic acid expression

construct encoding an upstream activator of the conditionally active transactivation domain.

27. A method of assaying for the interaction of two proteins in a mammalian cell, said method comprising the steps of:

(I) detecting in a mammalian cell expression of a reporter gene, wherein said mammalian cell contains in its genome a stably integrated recombinant reporter gene construct comprising a reporter gene operably linked to one or more copies of a sequence-specific recognition site, and said mammalian cell comprises (a) a recombinant nucleic acid construct encoding a first protein fused to a DNA binding domain that specifically binds said sequence-specific recognition site; (b) a recombinant nucleic acid construct encoding a second protein fused to an activation domain of a transcriptional activator protein, wherein said activation domain transactivates expression of a gene to which it is bound; and

II) detecting the expression of said reporter gene, wherein said expression indicates interaction of said first protein and said second protein in said mammalian cell.

28. A method of screening for a modulator of the interaction of two proteins known to interact in a mammalian cell, said method comprising the steps of:

i) contacting a mammalian cell with a candidate modulator, said mammalian cell containing in its genome a stably integrated reporter gene construct which comprises a reporter gene operably linked to one or more copies of a sequence-specific recognition site, and said mammalian cell also comprises

(a) a nucleic acid construct encoding a first protein of an interaction pair fused to a

DNA binding domain which specifically binds said sequence-specific recognition site; and

(b) a nucleic acid construct encoding a second protein of an interaction pair fused to an activation domain of a transcriptional activator protein, wherein said activation domain transactivates expression of a gene to which it is bound; and

ii) detecting the expression of said reporter gene, wherein a difference in reporter gene expression in the presence and absence of said candidate modulator is indicative of modulation by said candidate modulator of the interaction of said first and second proteins in said mammalian cell.

29. The method of either one of claims 27 or 28 wherein said DNA binding protein or DNA binding domain thereof is selected from the group consisting of GAL4 and LexA.

30. The method of either one of claims 27 or 28 wherein said DNA binding protein or DNA binding domain thereof is GAL4.

31. The method of either one of claims 27 or 28 wherein said mammalian cell is derived from a human.

32. The method of claim 31 wherein said mammalian cell is a HeLa cell.

COMPOSITIONS AND METHODS UTILIZING STABLE REPORTER CELL LINES FOR
DETECTION OF PATHWAY-SPECIFIC SIGNAL TRANSDUCTION

ABSTRACT OF THE DISCLOSURE

The invention encompasses compositions and methods which utilize a cell line comprising a stably integrated recombinant nucleic acid construct comprising a reporter gene operably linked to a recognition sequence for a sequence-specific DNA-binding protein and a stably integrated recombinant nucleic acid construct comprising a sequence encoding a fusion protein, the fusion protein comprising a sequence-specific DNA binding domain, wherein the DNA binding domain specifically binds the recognition sequence, and a conditionally active transactivation domain, wherein activation of the conditionally active transactivation domain is dependent on protein phosphorylation and/or protein:protein interaction, and wherein binding of the fusion protein to the recognition sequence results in transactivation of the reporter gene expression when the transactivation domain fused to the DNA binding domain is activated.

Figure 1

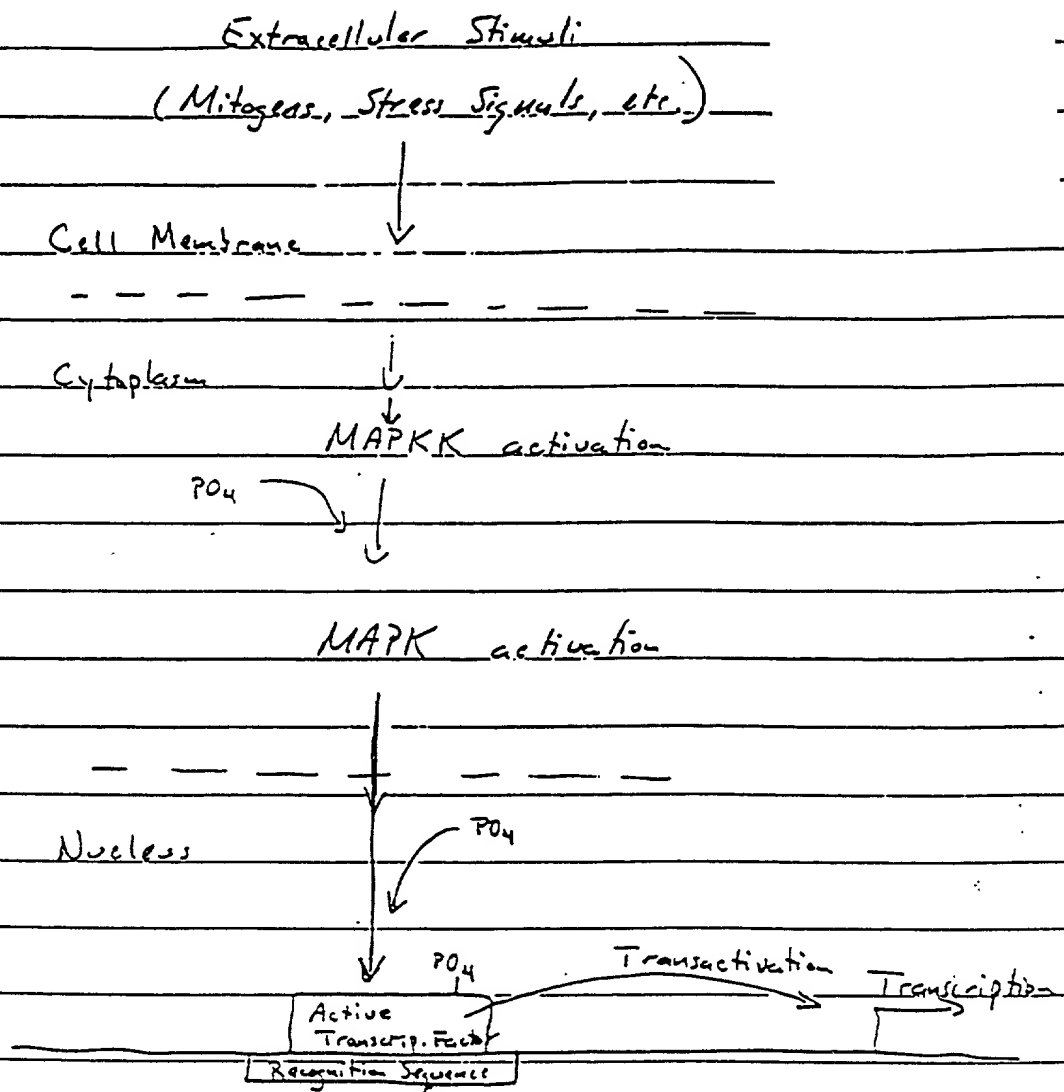


Figure 2

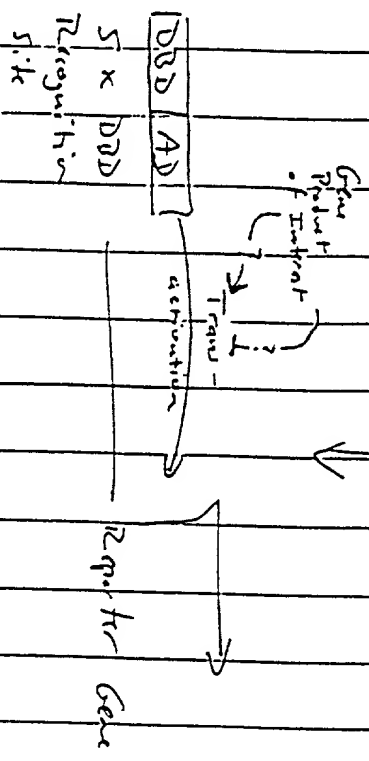
Mastering Pathway - Specific Signal Transduction

Transcriptional Planit + Regulator Planit +

DBD: Relaying Specific AD - 5x DBD - Regulator Gene -

Gene of Interest Planit
Promoter - Gene of Interest

Translating Co-Transfect



00037650 0001100

Common

Figure 3

Selection of Stable Reporter Cell Lines

Reporter Construct (w/ linked DBD element)



Stably transfect



Screen for clones with low background
of Reporter activity and strong
response to DBD-bearing activator(s)



"Stable Reporter Cell Line"



Stably transfect with fusion
transactivator plasmid



Screen for clones with strong
response to pathway-specific
upstream activator(s)



"Pathway-Specific Stable Reporter Cell Line"

Figure 4

4.1.1. pFR-Luc Plasmid



Sequence of GAL4 Binding Element in the pFR-Luc Plasmid

```

GT CGGAGTACTGTCCTCCG AG CGGAGTACTGTCCTCCG
AG CGGAGTACTGTCCTCCG AG CGGAGTACTGTCCTCCG
AG CGGAGTACTGTCCTCCG AG CGGAGACTCTAGAGGG
TATATATGGATCCCCGGGT AC CGAGCTCGAATTC--
--CAGCTTGGCATTCCGGTACTGTTGGTAAATG--Luciferase
  
```

001180"0594E960

4.1.4. pFA-CMV Plasmid

pFA-CMV Plasmid

BamHI | SrfI | SmaI | EcoRI | XbaI | HindIII | PstI | SacI | KpnI | BglII
 GTA TCG CCG GGA TCG GCG CGG GCT GGA ATT CTA GAA GGT TCT GCA GAG CTC GGT ACC AGA TCT TGA ATA AGT AG
 V S P G S G R A G I L E A S A E L G T R S * * *

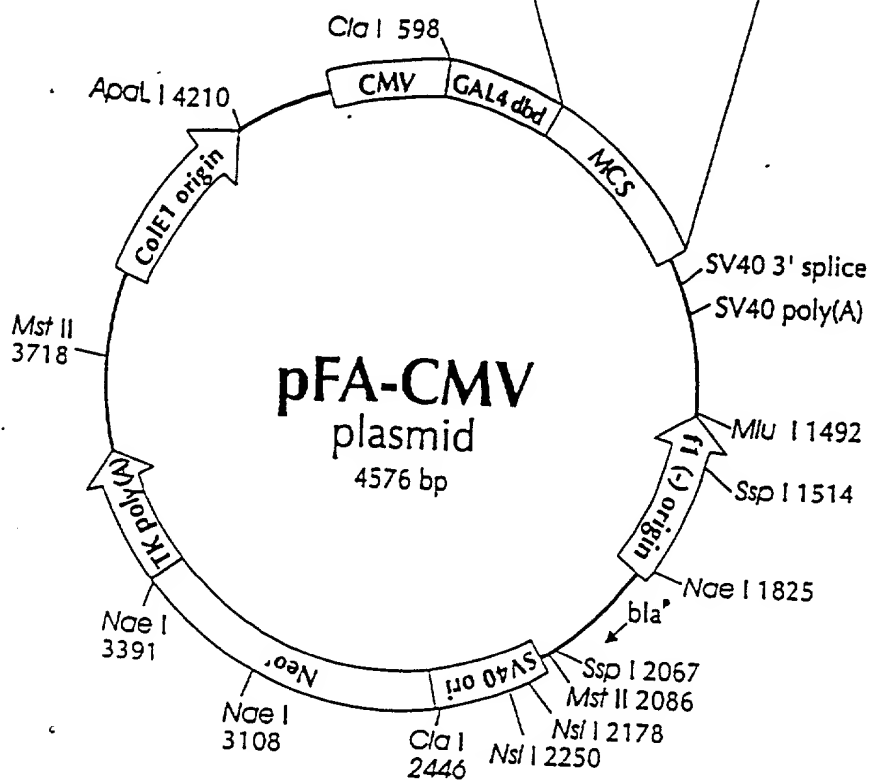


Figure 5

09637650.081100

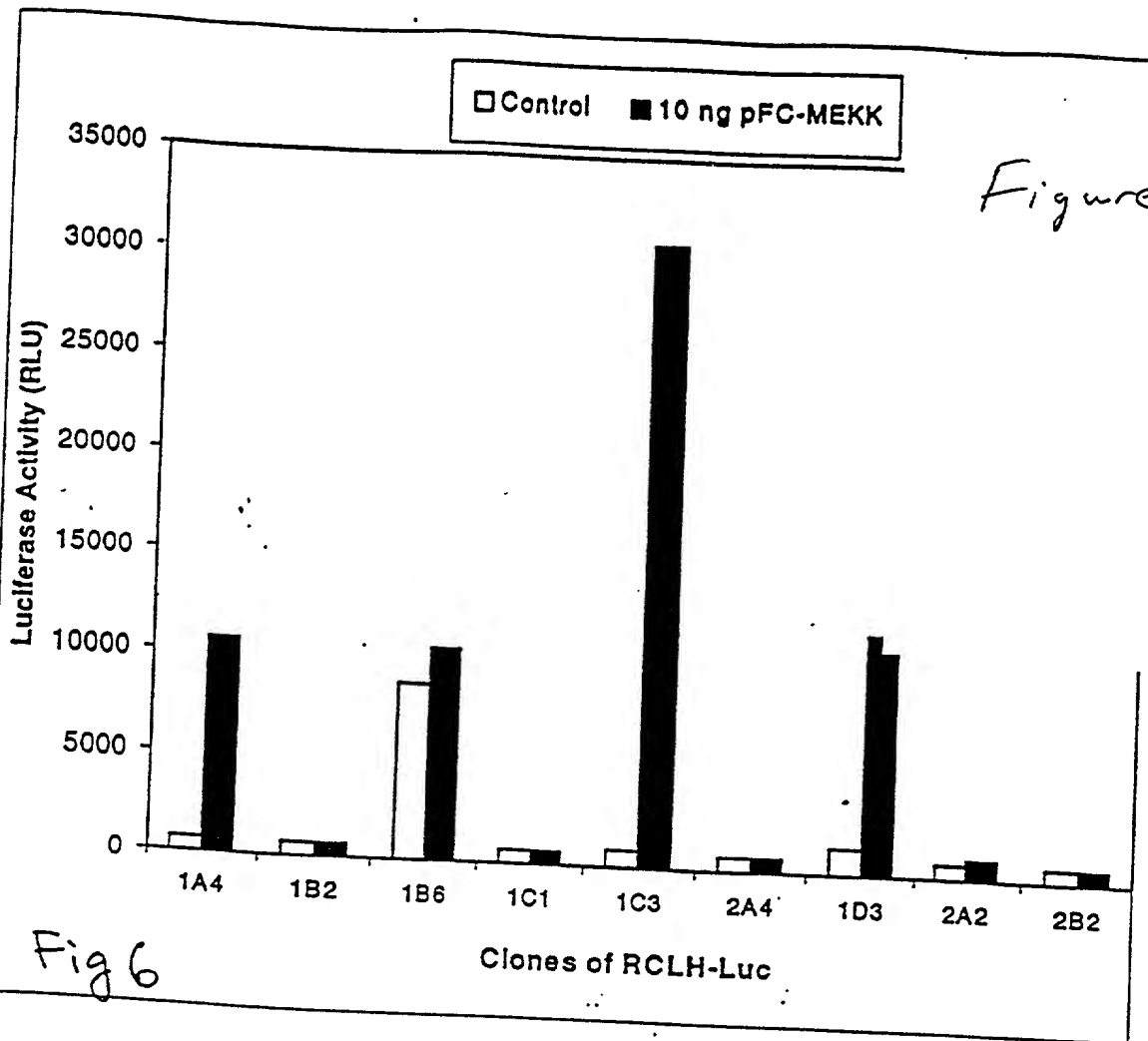


Figure 7

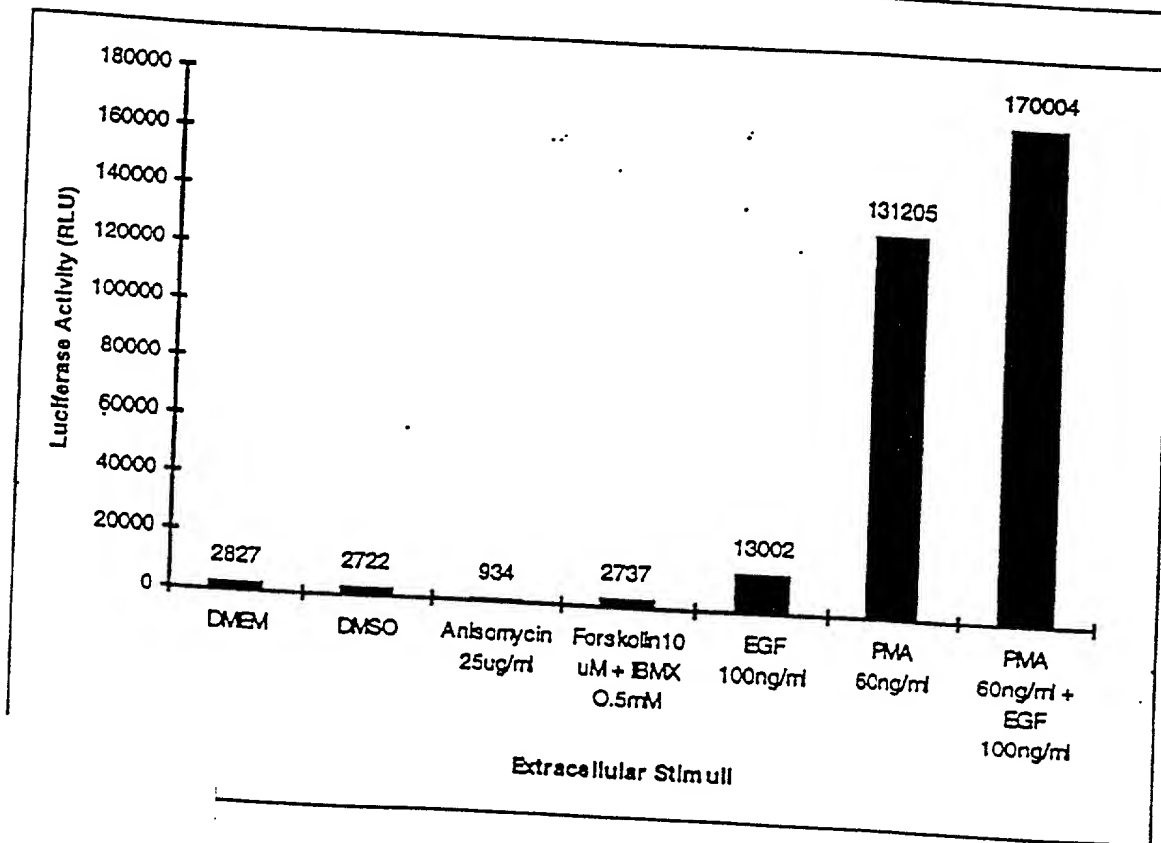
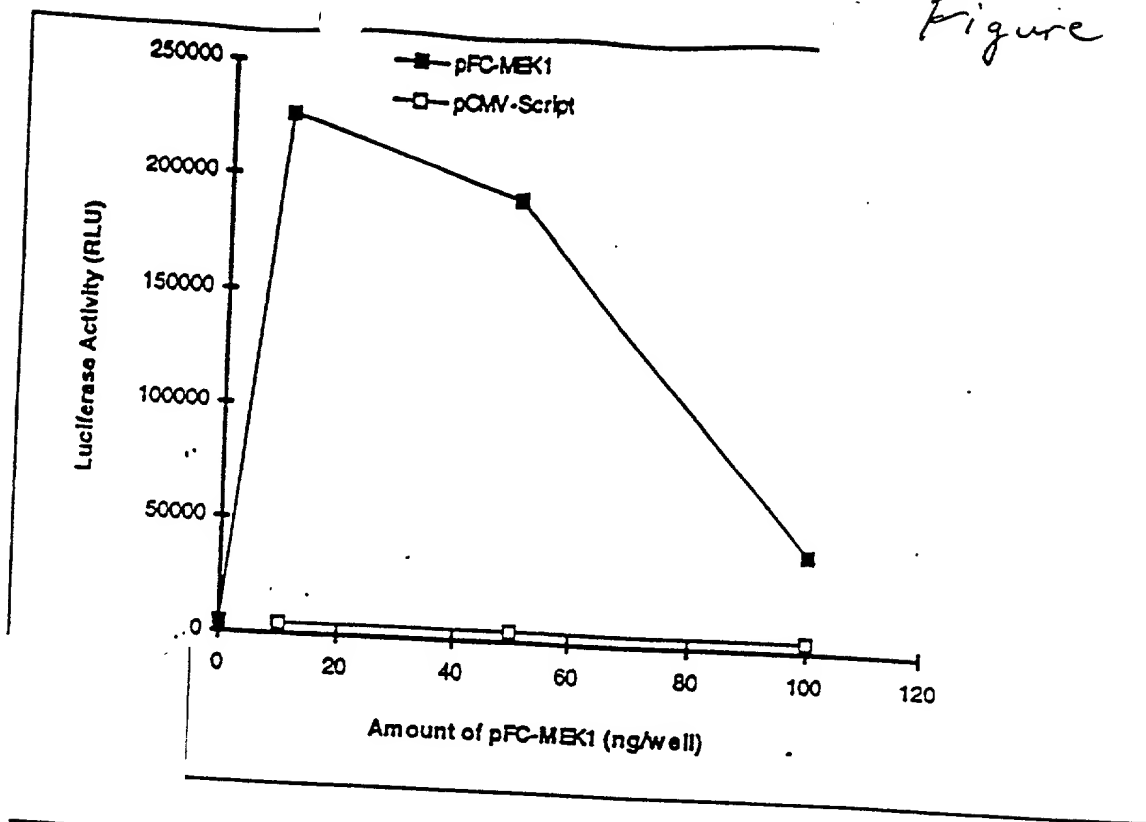
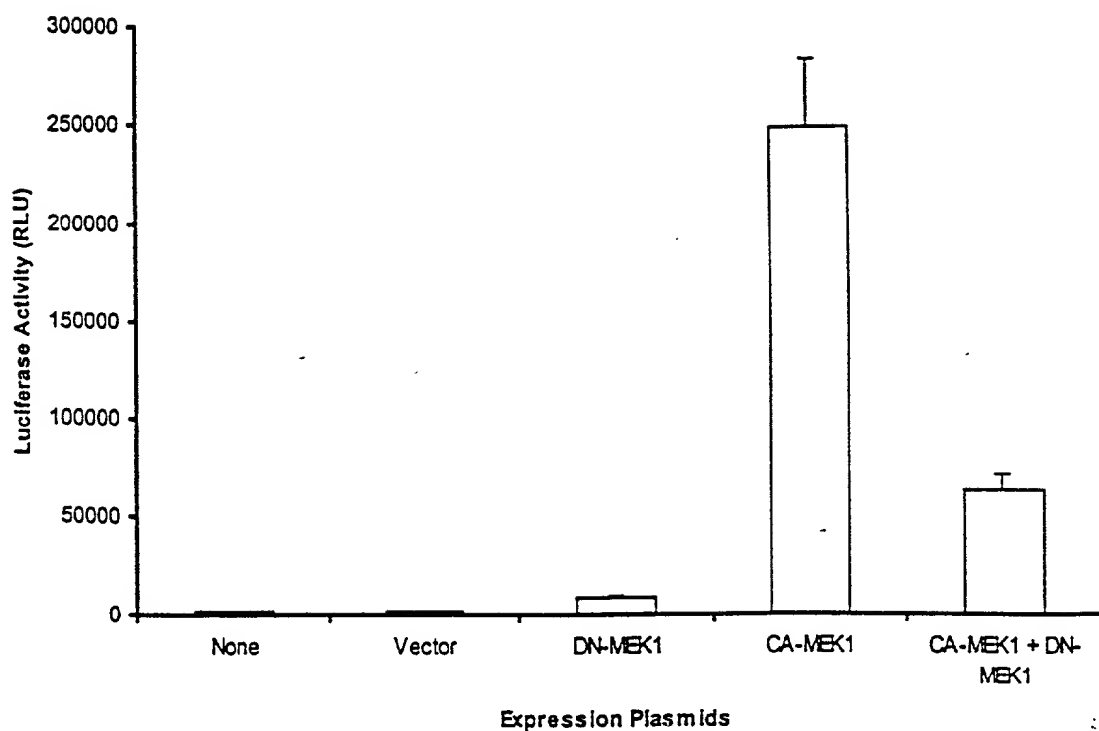


Figure 8

001180"0532E960

Figure



123/

Figure 9

00780-0592E960

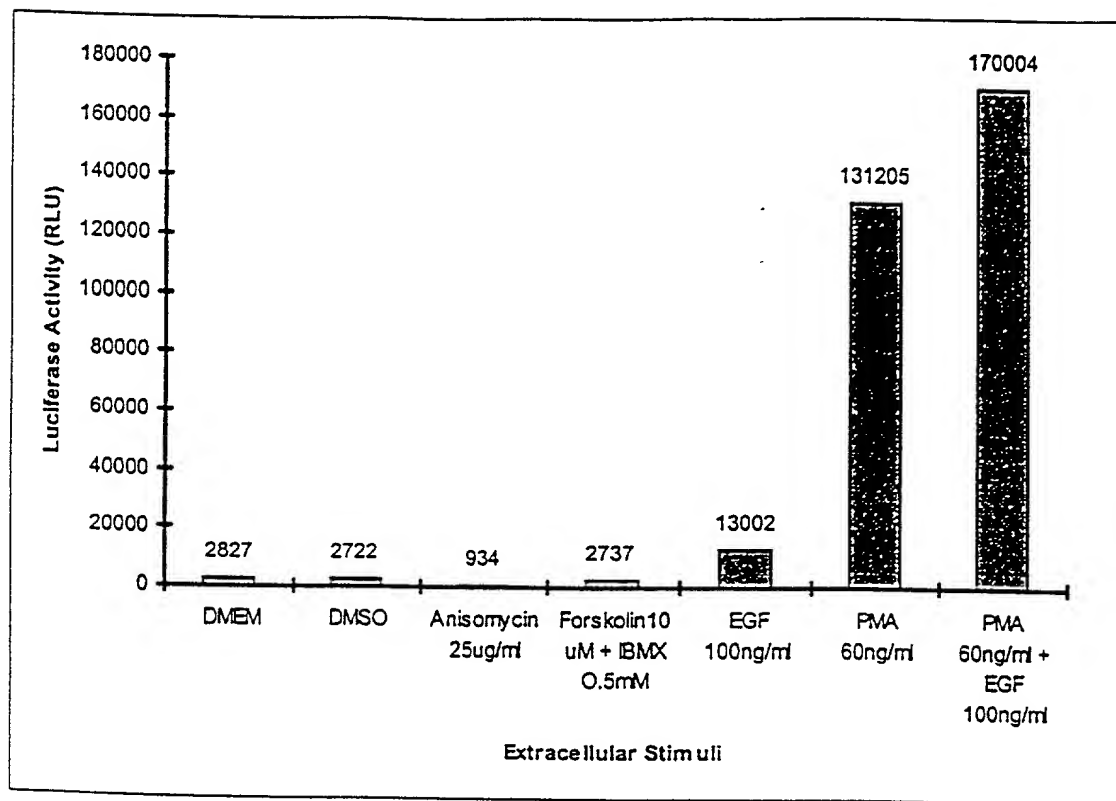


Figure 10

DOTED 06/25/98

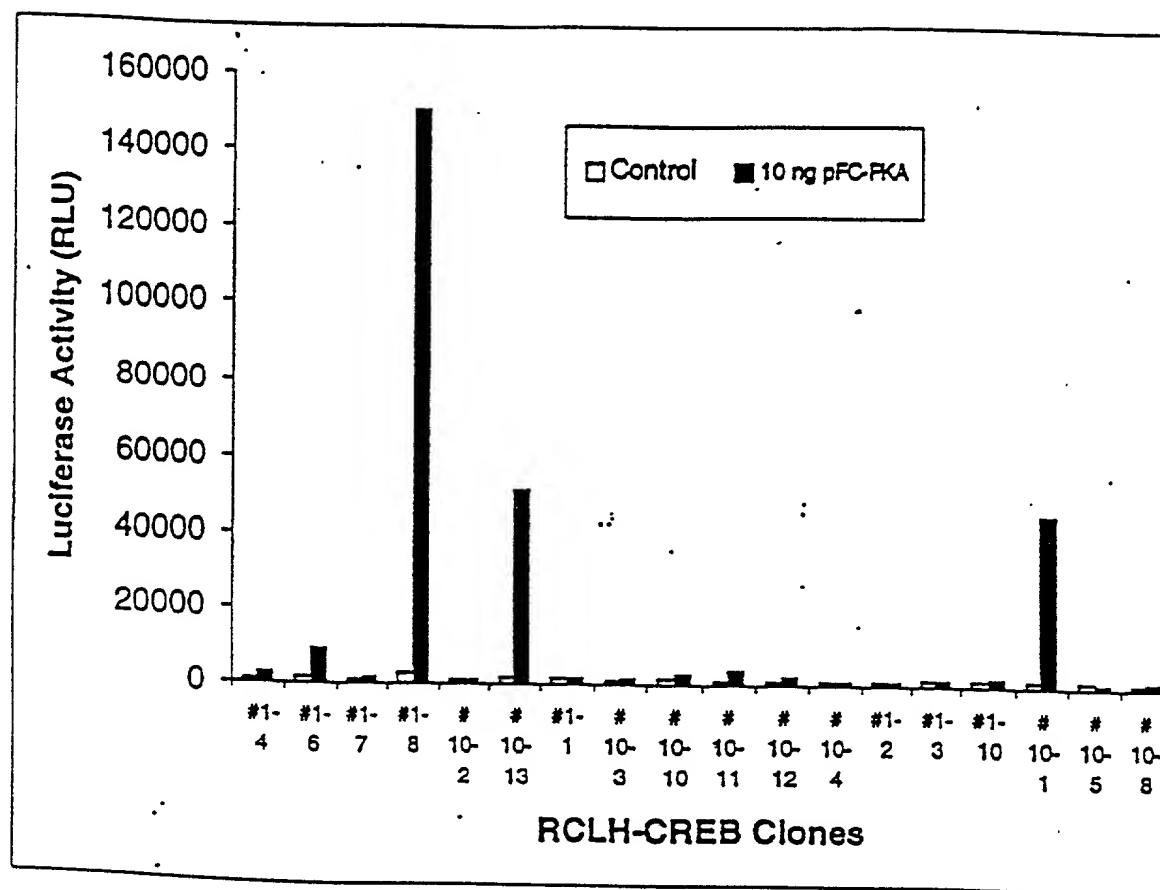


Figure 11

Figure 12

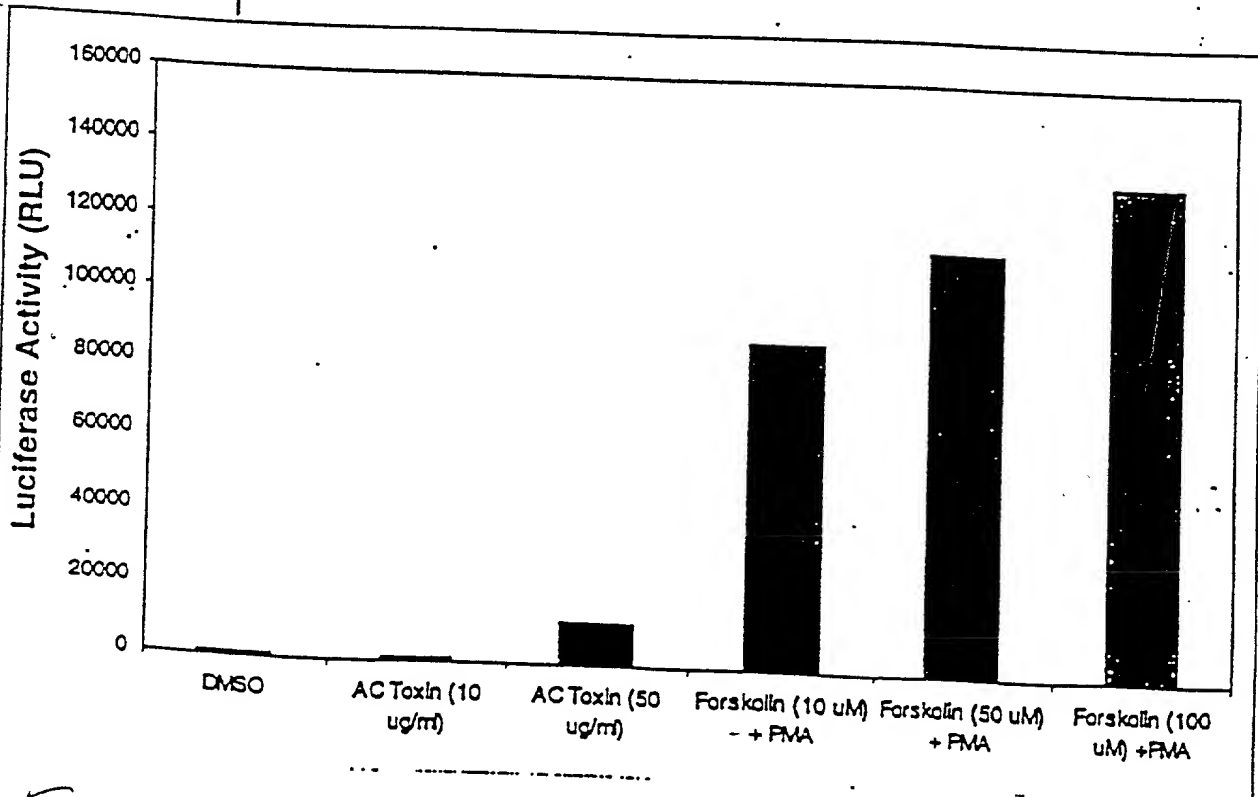
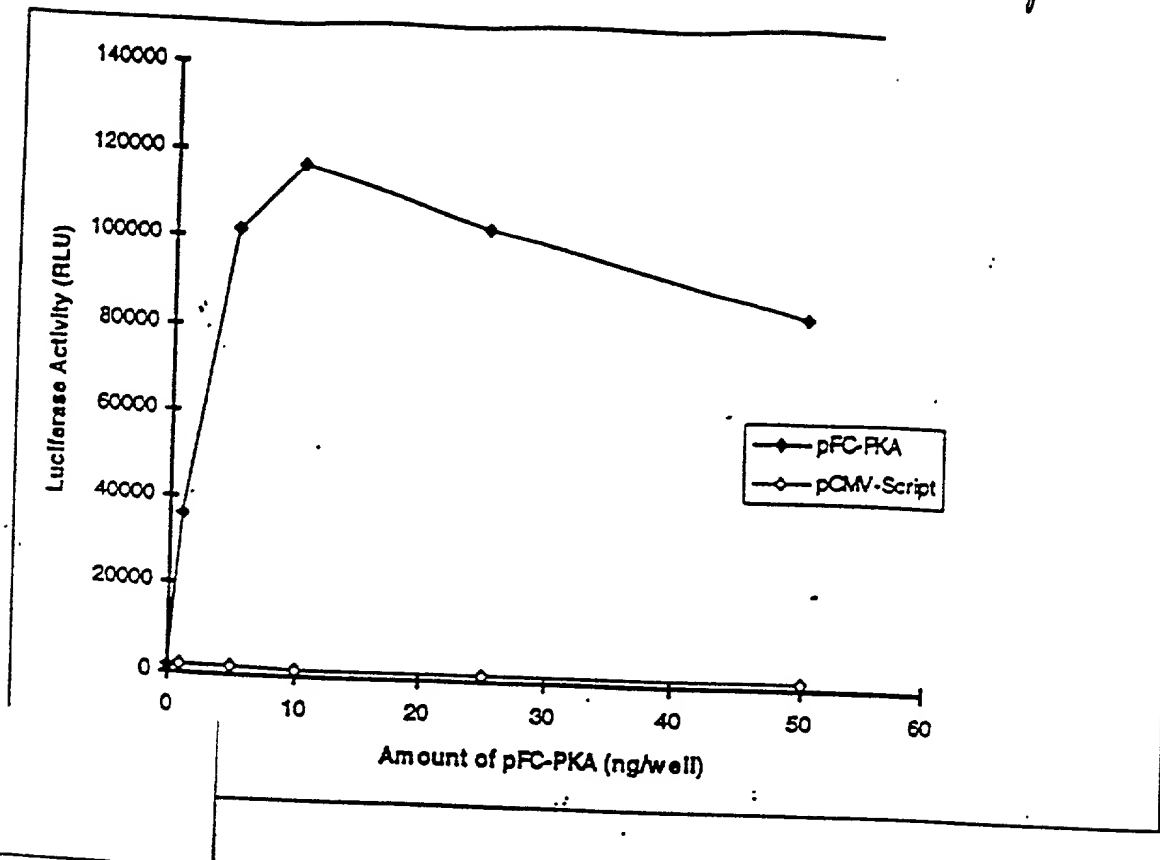


Figure 13

00780 0594E960

001130 092550

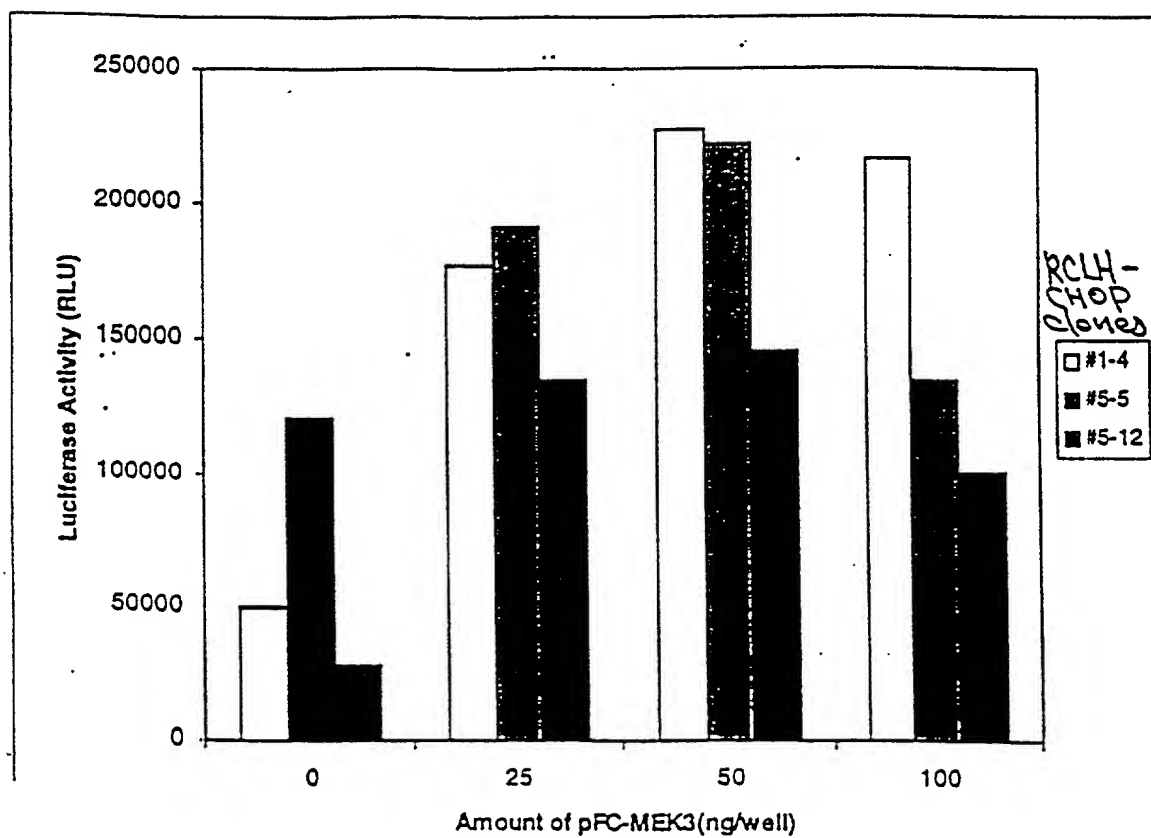


Figure 14

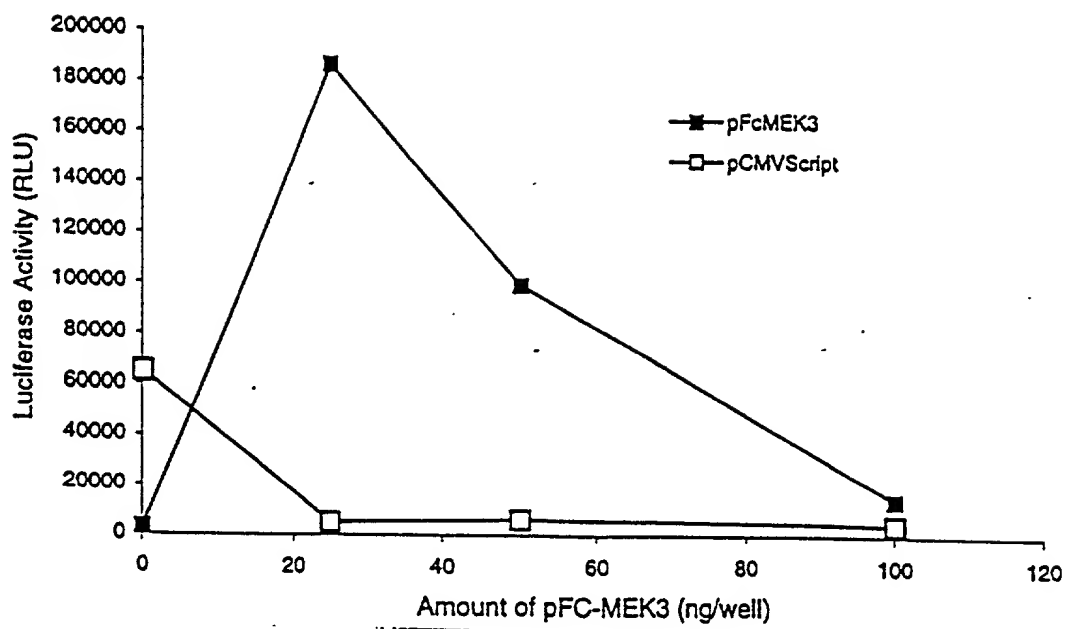


Figure 15

00780" 0592E960

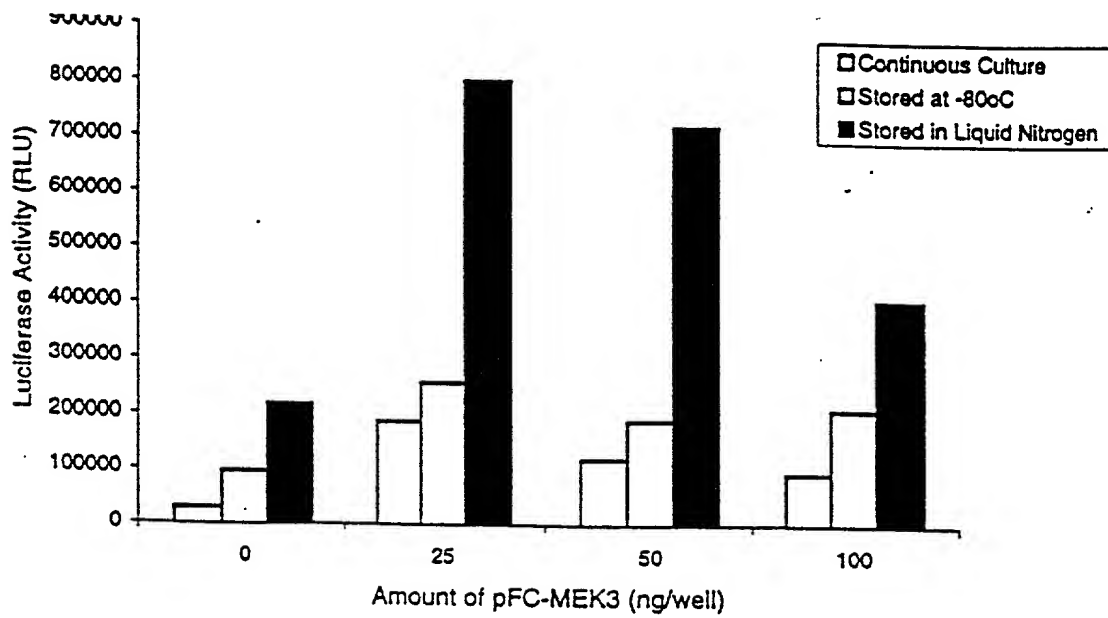


Figure 16

007730 039/350

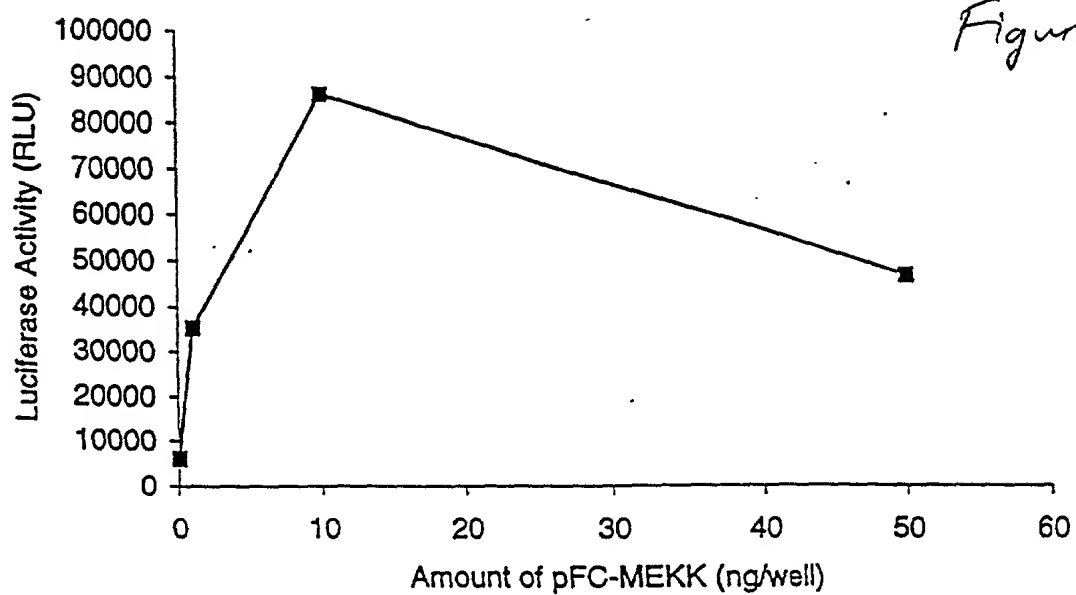
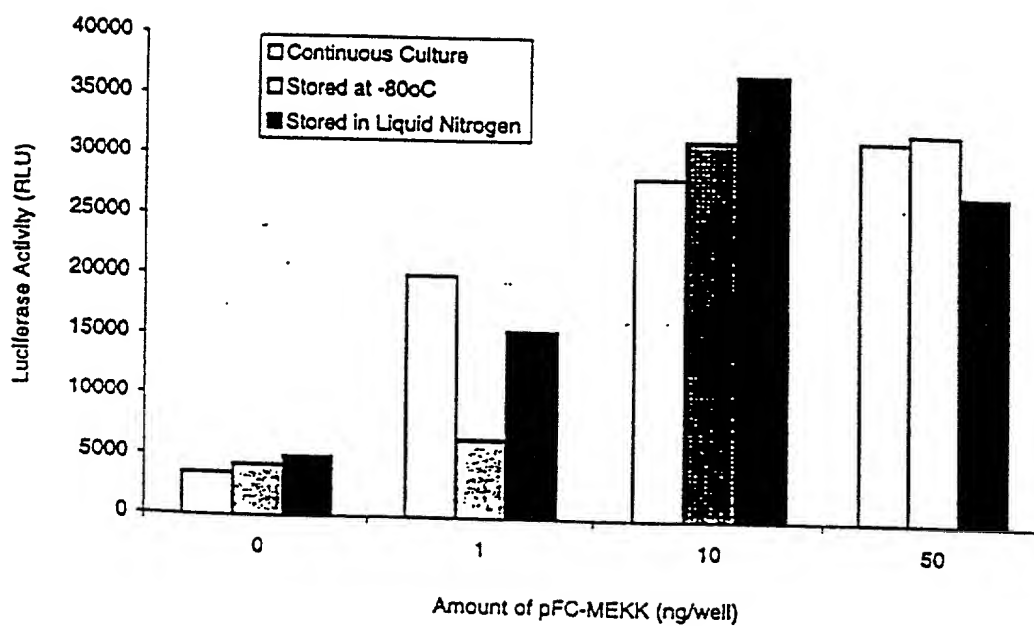


Figure 17

00780 0592E960

Figure 18



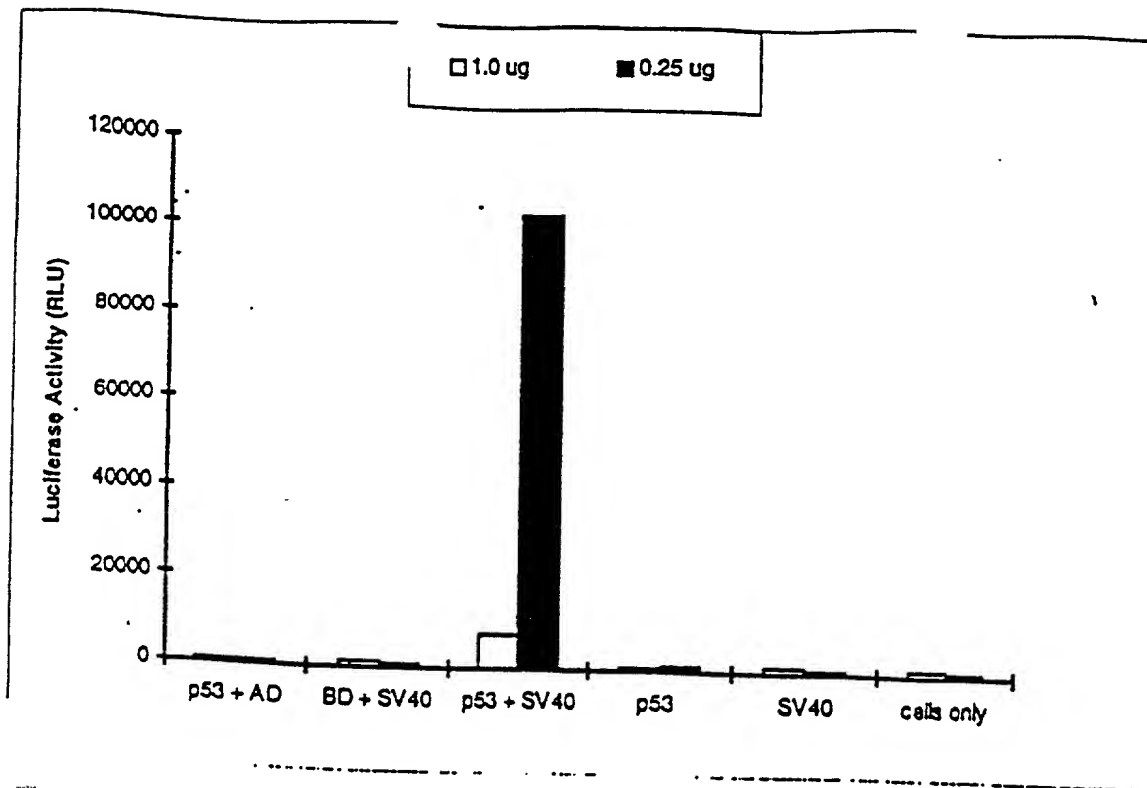


Figure 19

001130" 053/1E960

DECLARATION AND POWER OF ATTORNEY
(FOR PATENT APPLICATION)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below, next to my name.

I believe that I am an original, first and joint inventor of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

**Compositions and Methods Utilizing Stable Reporter Cell Lines for Detection of Pathway-Specific
Signal Transduction**

the specification of which is attached hereto .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I do now know and do not believe that the invention was ever patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to this application.

I do not know and do not believe that the invention was in public use or on sale in the United States of America more than one year prior to this application.

I acknowledge the duty to disclose information, which is material to the examination of this application in accordance with Title 37 CFR § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which prior is claimed:

Prior Foreign Application(s):

			Priority Claimed [] []
(Number)	(Country)	(Day/Month/Year filed)	

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

	<u>Application Number</u>	<u>Filing Date</u>
1.	60/150,902	August 26, 1999

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

	<u>Application Number</u>	<u>Filing Date</u>	<u>Status - patented, pending, abandoned</u>
1.			

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

APPOINTED PRACTITIONER(S)	REGISTRATION NUMBER(S)
Kathleen M. Williams	34,380
Richard B. Smith	34,020
Elizabeth Neuman Spar	45,123
Paula Campbell Evans	32,503
Mark J. FitzGerald	45,928
John M. Garvey	37,833

Address all correspondence to:

Kathleen M. Williams, Ph.D., Esquire
Palmer & Dodge, LLP
One Beacon Street
Boston, MA 02108

Direct all telephone calls to **Kathleen M. Williams, Ph.D., Esquire** at (617) 573-0451)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

First Named Inventor:

Date: _____

Inventor's Signature

Chao-Feng Zheng

Residence: United States

Citizenship: United States

Post Office Address: 11641 Trailbrush Point, San Diego, California

ASSIGNMENT

Case No.: 25436/1510
Inventors: Chao-Feng Zheng
Filing Date:

Serial No.:
Date of Execution:

In consideration of One Dollar (\$1.00) and other good and valuable considerations in hand paid, the receipt and sufficiency whereof are hereby acknowledged, the undersigned hereby assigns to: Stratagene, a California corporation, having a principal place of business at 11011 North Torrey Pines Road, LaJolla, CA 92037, its successors and assigns, the entire right, title and interest in the invention or improvements of the undersigned disclosed in an application for Letters Patent of the United States, entitled:

COMPOSITIONS AND METHODS UTILIZING STABLE REPORTER CELL LINES FOR DETECTION OF PATHWAY-SPECIFIC SIGNAL TRANSDUCTION

and identified as:

U.S. Serial Number:

and in said application and any and all other applications, both United States and foreign, which the undersigned may file, either solely or jointly with others, on said invention or improvements, and in any and all Letters Patent of the United States and foreign countries, which may be obtained on any of said applications, and in any reissue or extension of such patents, and further assigns to said assignee the priority right provided by the International Convention.

The undersigned hereby authorizes and requests the Commissioner of Patents and Trademarks to issue said Letters Patent to said assignee.

The undersigned warrants himself to be the owner of the entire right, title and interest in said invention or improvements and to have the right to make this assignment, and further warrant that there are no outstanding prior assignments, licenses, or other encumbrances on the interest herein assigned.

For said considerations the undersigned hereby agrees, upon the request and at the expense of said assignee, its successors and assigns, to execute any and all divisional, continuation and substitute applications for said invention or improvements, and any necessary oath, affidavit or declaration relating thereto, and any application for the reissue or extension of any Letters Patent that may be granted upon said application and any and all applications and other documents for Letters Patent in foreign countries on said invention or improvements, that said assignee, its successors or assigns may deem necessary or expedient, and for the said considerations the undersigned authorize said assignee to apply for patents for said invention or improvements in its own name in such countries where such procedure is proper and further agree, upon the request of said assignees, its successors and assigns, to cooperate to the best of the ability of the undersigned with said assignee, its successors and assigns, in any proceedings or transactions involving such applications or patents, including the preparation and execution of preliminary statements, giving and producing evidence, and performing any and all other acts necessary to obtain, maintain and enforce said Letters Patent, both United States and foreign, and vest all rights therein hereby conveyed in the assignees, its successors and assigns, whereby said Letters Patent will be held and enjoyed by the said assignees, its successors and assigns, to the full end of the term for which said Letters Patent will be granted, as fully and entirely as the same would have been held and enjoyed by the undersigned if this assignment had not been made.

001180-0592560

IN WITNESS WHEREOF, we have hereunto set our hands and affixed our seals.

Chao-Feng Zheng

Date: _____

County of

) SS.

)

Subscribed before me by the above-named Chao-Feng Zheng this ____ day of _____, 2000.

Notary

My commission expires: _____

Table 1. Demographic characteristics of the study population	
Age (years)	Mean (SD)
18-24	20.5 (2.5)
25-34	29.5 (4.5)
35-44	39.5 (5.5)
45-54	49.5 (6.5)
55-64	59.5 (7.5)
65-74	69.5 (8.5)
75-84	79.5 (9.5)
85-94	89.5 (10.5)
95-104	99.5 (11.5)
105-114	109.5 (12.5)
115-124	119.5 (13.5)
125-134	129.5 (14.5)
135-144	139.5 (15.5)
145-154	149.5 (16.5)
155-164	159.5 (17.5)
165-174	169.5 (18.5)
175-184	179.5 (19.5)
185-194	189.5 (20.5)
195-204	199.5 (21.5)
205-214	209.5 (22.5)
215-224	219.5 (23.5)
225-234	229.5 (24.5)
235-244	239.5 (25.5)
245-254	249.5 (26.5)
255-264	259.5 (27.5)
265-274	269.5 (28.5)
275-284	279.5 (29.5)
285-294	289.5 (30.5)
295-304	299.5 (31.5)
305-314	309.5 (32.5)
315-324	319.5 (33.5)
325-334	329.5 (34.5)
335-344	339.5 (35.5)
345-354	349.5 (36.5)
355-364	359.5 (37.5)
365-374	369.5 (38.5)
375-384	379.5 (39.5)
385-394	389.5 (40.5)
395-404	399.5 (41.5)
405-414	409.5 (42.5)
415-424	419.5 (43.5)
425-434	429.5 (44.5)
435-444	439.5 (45.5)
445-454	449.5 (46.5)
455-464	459.5 (47.5)
465-474	469.5 (48.5)
475-484	479.5 (49.5)
485-494	489.5 (50.5)
495-504	499.5 (51.5)
505-514	509.5 (52.5)
515-524	519.5 (53.5)
525-534	529.5 (54.5)
535-544	539.5 (55.5)
545-554	549.5 (56.5)
555-564	559.5 (57.5)
565-574	569.5 (58.5)
575-584	579.5 (59.5)
585-594	589.5 (60.5)
595-604	599.5 (61.5)
605-614	609.5 (62.5)
615-624	619.5 (63.5)
625-634	629.5 (64.5)
635-644	639.5 (65.5)
645-654	649.5 (66.5)
655-664	659.5 (67.5)
665-674	669.5 (68.5)
675-684	679.5 (69.5)
685-694	689.5 (70.5)
695-704	699.5 (71.5)
705-714	709.5 (72.5)
715-724	719.5 (73.5)
725-734	729.5 (74.5)
735-744	739.5 (75.5)
745-754	749.5 (76.5)
755-764	759.5 (77.5)
765-774	769.5 (78.5)
775-784	779.5 (79.5)
785-794	789.5 (80.5)
795-804	799.5 (81.5)
805-814	809.5 (82.5)
815-824	819.5 (83.5)
825-834	829.5 (84.5)
835-844	839.5 (85.5)
845-854	849.5 (86.5)
855-864	859.5 (87.5)
865-874	869.5 (88.5)
875-884	879.5 (89.5)
885-894	889.5 (90.5)
895-904	899.5 (91.5)
905-914	909.5 (92.5)
915-924	919.5 (93.5)
925-934	929.5 (94.5)
935-944	939.5 (95.5)
945-954	949.5 (96.5)
955-964	959.5 (97.5)
965-974	969.5 (98.5)
975-984	979.5 (99.5)
985-994	989.5 (100.5)
995-1004	999.5 (101.5)
1005-1014	1009.5 (102.5)
1015-1024	1019.5 (103.5)
1025-1034	1029.5 (104.5)
1035-1044	1039.5 (105.5)
1045-1054	1049.5 (106.5)
1055-1064	1059.5 (107.5)
1065-1074	1069.5 (108.5)
1075-1084	1079.5 (109.5)
1085-1094	1089.5 (110.5)
1095-1104	1099.5 (111.5)
1105-1114	1109.5 (112.5)
1115-1124	1119.5 (113.5)
1125-1134	